Plants as Bioreactors:

Expression of *Toxoplasma gondii* Surface Antigen P30 in Transgenic Tobacco Plants

By

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Statement

All experimental works reported in this thesis were performed by the author, unless specially stated otherwise in the text.

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Abstract

Toxoplasma gondii (*T. gondii*) is a widespread, obligate intracellular protozoan parasite with an exceptional ability to invade, survive and replicate within nearly all nucleated cells. It is responsible for toxoplasmosis in warm-blooded vertebrates. Although generally mild or asymptomatic for healthy people, toxoplasmosis may cause abortion or neonatal malformations if contracted during pregnancy. Furthermore, it is often lethal for immunocompromised patients and transplant recipients. Many studies have been focused on improvement of diagnostic testing for toxoplasmosis and development of subunit vaccines that could prevent or attenuate the disease.

P30, a major surface antigen of *T. gondii*, is very abundant, conserved in most strains, and present both in the vesicular network of the parasitophorous vacuole and on the surface of the parasite. It is highly immunogenic and can stimulate organisms to elicit IgG, IgM, IgA antibodies and interferon γ. Purified P30 has been tested as a single antigen for serodiagnosis of acute and chronic toxoplasmosis. Also, passive transfer of monoclonal anti-P30 antibody and active immunization with P30 antigen in liposomes suggested that P30 is a protective immunogen against toxoplasmosis. The gene encoding for P30 has been cloned and completely

sequenced. Therefore, it would be valuable to produce large amount and high quality P30 by recombinant DNA technology for applications such as diagnostic reagent and potential subunit vaccine, and for study on its role in invasion.

To test whether plants can be used as bioreactors to produce high quality *T. gondii* surface antigen P30 in bulk amount, the gene encoding this antigen, under the control of constitutive or seed-specific promoter, had been introduced into tobacco. Successful integration of transgenes into tobacco genome was confirmed by genomic PCR and Southern blot analysis. The presences of P30 transcripts were detected by RT-PCR and northern blot analysis. However, no recombinant P30 protein could be detected by SDS-PAGE and western blot analysis. Possible reasons for failure in P30 expression in plants may be sited at translation and/or posttranslation levels. Further studies are proposed and required to improve P30 translation efficiency and protein stability in plants.

摘要

原生動物弓形蟲 (*Toxoplasma gondii*) 是一種常見的專性胞內寄生蟲。 它的寄主範圍很廣，能侵入大部份有核細胞並能在內生存及複制。 而恆溫動物中的弓漿蟲病 (toxoplasmosis) 亦爲弓形蟲所引起。 弓漿蟲病對健康人而言通常是影響輕微或無病徵，但若在妊娠期間患上則可能導致流產或畸形嬰兒。 此外，失去免疫能力及接受器官移植的病人亦時常因感染此病而死亡。 現在有關弓形蟲的研究着重在怎樣改善診斷弓漿蟲病的方法及發展能預防或減低病害的亞單位疫苗。

P30 是弓形蟲主要表面抗原。 P30 含量豐富，結構保守，能並存於寄主液泡的具囊網絡及自身的表面。 P30 具有高度的免疫原性，能誘發生物體免疫球蛋白 G、免疫球蛋白 M、免疫球蛋白 A 及γ-干擾素。 實驗已証明，純化的 P30 能在急性及慢性弓漿蟲病的血清學診斷中作爲單一抗原。 此外，在老鼠中 P30 單克隆抗體的被動轉化及脂質體的 P30 抗原主動免疫亦能說明 P30 是對抗弓漿蟲病的保護性免疫原。 現在 P30 的基因已被克隆，其完整序列亦知，若我們能通過 DNA 重組工程大量生產高質素的 P30 蛋白作診斷試劑、亞單位疫苗或入侵寄主途徑的研究，將會有重大的價值。

本研究的重點是嘗試利用植物作爲生物反應器去大量生產高質素的弓形蟲表面抗原 P30。 在本實驗中，編碼 P30 的基因與組成型表達或種子特異性表達的啓動子連接構建成嵌合基因並轉化煙草。 基因組 PCR 及 Southern 雜交的分析均証明嵌合基因經整合後已成功進入煙草的基因組中。 而逆轉錄 PCR 及

northern 雜交的分析亦顯示了 P30 mRNA 的存在。 然而，通過 SDS-PAGE 及 western 雜交，我們未能發現任何 P30 蛋白產物。 P30 未能在植物中表達的原因可能在於翻譯及/或後翻譯水平上。 我們對如何提高 P30 在植物中的翻譯效率及蛋白穩定性提出建議及作進一步的研究。

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List of Abbreviations

+ve	Positive
β-mer	β-mercaptoethanol
6xHis	Hexa-histidine
A	Adenine
a. a.	Amino acids
a. a. mix-Leu	Amino acids mixture minus leucine
a. a. mix-Met	Amino acids mixture minus methionine
Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartate
BA	N^6-benzyladenine
BCA	Bicinochoninic acid
BMV	Brome Mosaic Virus
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CaMV	Cauliflower mosaic virus /viral
Car	Carbenicillin
cDNA	Complementary DNA
CMV	Cowpea mosaic virus
CSPD	Disodium 3-(4-methoxyspirol{1,2-dioewtane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	Cetyltrimethylammonium bromide
DAF	Day-after-flower
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EK	Enterokinase
ER	Endoplasmic reticulum
EtOH	Ethanol
G	Guanine
GFP	Green fluorescent protein
Gln (Q)	Glutamine
Glu (E)	Glutamate
Gly (G)	Glycine
GPI	Glycosyl-phosphatidylinositol
GUS	β-glucuronidase
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
His (H)	Histidine
His-tag	Hexa-histidine tag
Ig	Immunoglobulin
Ile (I)	Isoleucine
iscom	immunostimulating complexes
Kan	Kanamycin
kb	Kilobases
kDa	Kilodaltons
LB	Left border (of T-DNA in pBI121) or Luria-Bertani (medium)
Leu (L)	Leucine
LRP	Lysine-rich protein
Lys (K)	Lysine
M	Molar
MCS	Multiple cloning site
MES	2-(morpholino)ethanesulfonic acid

Met (M)	Methionine
MEV	Mink enteritis virus
M-MLV	Moloney murine leukemia virus
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS	Murashige and Skoog (medium)
MW	Molecular weight
Ni-NTA	Nickel-nitrilotriacetic acid
Nos	Nopaline synthase
NPTII	Neomycin phosphotransferase II
NVCP	Norwalk virus capsid protein
OD	Optical density (absorbance)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Phas	Phaseolin
Phe (F)	Phenylalanine
PN2S	methionine-rich 2S albumin from Parasite nut
pro	Promoter
Pro (P)	Proline
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene difluoride
RB	Right border (of T-DNA of pBI121)
RL	Rabbit Reticulocyte Lysate
RNA	Ribonucleic acid
RNase	Ribonuclease
rNTP	Ribonucleotide triphosphate
rNVCP	Recombinant Norwalk virus capsid protein
rpm	Revolution per minute
RT-PCR	Reverse transcription-polymerase chain reaction
SAG1	Surface antigen 1
SDS	Sodium dodecyl sulfate

Ser (S)	Serine
SP	Signal peptide
SSC	Standard saline citrate
T	Thymidine
TAE	Tris-acetate /EDTA
T-DNA	Transfer DNA
tRNA	Transfer RNA
TE	Tris-EDTA
Ter	Terminator
TGS	Transcriptional gene silencing
Thr (T)	Threonine
Ti-plasmid	Tumor-inducing plasmid
TPE	Total protein extract
Tris	Tris(hydroxymethyl)amminomethane
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Ubi	Maize ubiquitin 1
UV	Ultraviolet
v/v	Volume by volume
Val (V)	Valine
-ve	Negative
WG	Wheat Germ Extract
X-gluc	5-bromo-4-chloro-3-indolyl-β-glucuronic acid

Chapter 1 General Introduction

The protozoan *Toxoplasma gondii* is a widespread, obligate intracellular parasite. It has an extremely broad host range and an exceptional ability to invade, survive and replicate within nearly all nucleated cells (Tomavo *et al.*, 1992). Cats are the definitive hosts (Frenkel *et al.*, 1970) and are important in transmission of *T. gondii* to human beings and other animals, which serve as intermediate hosts. The life cycle of *T. gondii* is complex with two major asexual stages and a full sexual cycle (Dubey *et al.*, 1998).

T. gondii is responsible for toxoplasmosis in warm-blooded vertebrates. Approximately 30% of the world's human population is infected, although the prevalence varies markedly from country to country (Jackson and Hutchison, 1989). Generally mild or asymptomatic for healthy people, toxoplasmosis may cause abortion or neonatal malformations if contracted during pregnancy (Wong and Remington, 1994). Furthermore, it is often lethal for immunocompromised patients and transplant recipients (Frenkel, 1988). Many studies aim at improving diagnostic testing of toxoplasmosis and developing subunit vaccines that can prevent or attenuate the disease.

P30, also referred to as SAG1 (surface antigen 1), is a major surface antigen of *T. gondii*. It is a stage-specific antigen since it can be detected only in tachyzoite (Kasper *et al.*, 1984). It is very abundant (Santoro *et al.*, 1985), conserved in most strains (Ware and Kasper, 1987), and highly immunogenic (Partanen *et al.*, 1984; Khan *et al.*, 1988). Besides its important role in immune mechanism, P30 also involves in the first stages of invasion (Kasper and Khan, 1993).

With this information, it would be valuable to produce large amount and high quality of P30 by recombinant DNA technology, for production of diagnostic reagent and potential subunit vaccine, and for study on its role in invasion. A promising way to achieve this goal is to use plants as bioreactors or biochemical factories to the P30 protein. Using plants as bioreactors has the advantages including low cost, large quantity and high quality.

In this study, the gene encoding the *T. gondii* major surface antigen P30 was used to construct chimeric genes under the control of different promoters. These chimeric genes were delivered into tobacco genome via *Agrobacterium*-mediated transformation and their expression analysed. The ultimate goal is to produce large amounts and high quality of P30 for vaccination or for other aspects of study and utilization.

Chapter 2 Literature Review

2.1 *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan that belongs to the phylum Apicomplexa, class Sporozoa and subclass Coccidia. It is related to the parasites Plasmodium (the agents of malaria), Cryptosporidium, Eimeria, Isospora, Neospora and Sarcocystis. *T. gondii* has been recognized as one of the most common and successful parasites in the world by having an exceptional ability to invade, survive and replicate within nearly all nucleated cells of warm-blooded vertebrates (Tomavo *et al.*, 1992). It is so-called *Toxoplasma gondii* because of the characteristic crescent shape of its active tachyzoite form (toxoplasma means arc-shaped in Greek) and the wild rodent (*Ctenodactylus gondii*) from which it was first discovered in North Africa (Baker, 1969).

2.1.1 Morphology and Life Cycle of *T. gondii*

The life cycle of *Toxoplasma* is very complex. Though it is common in many animals, including rodents, birds, sheep, swine, and cattle, cats are the definitive hosts of *T. gondii* (Frenkel *et al.*, 1970). The sexual stage of *T. gondii* takes place only in the intestinal tract of cat (Figure 2- 1).

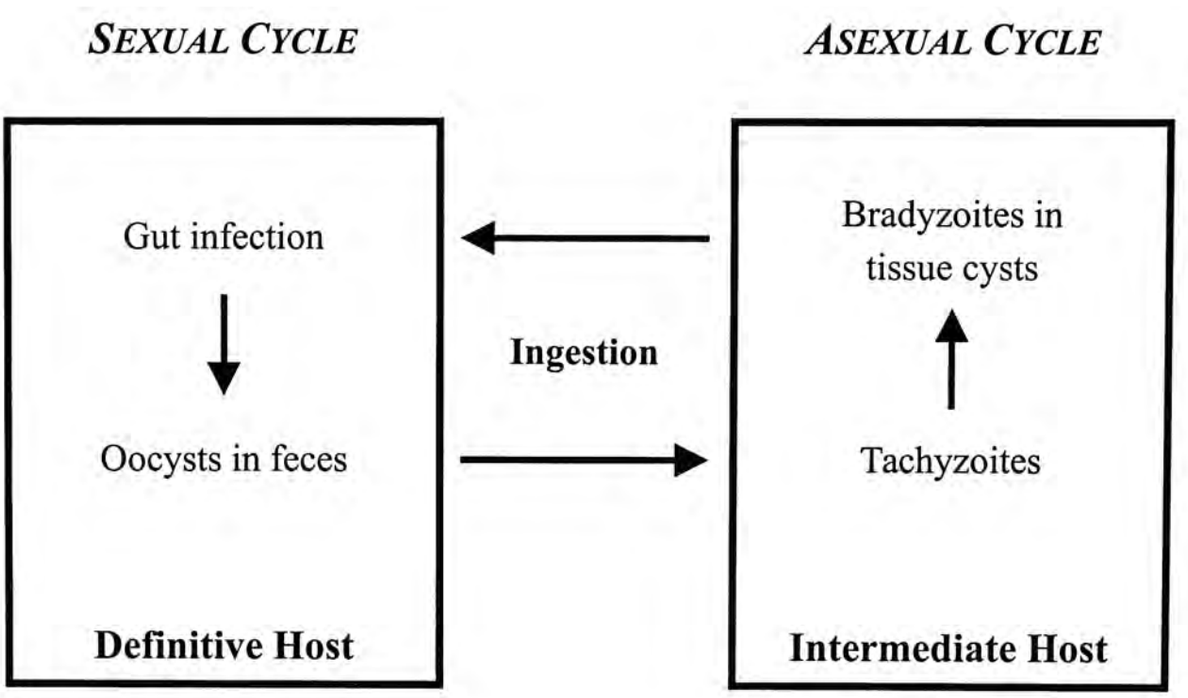


Figure 2- 1 The life cycle of *T. gondii*

There are three stages in a complete life cycle of *T. gondii* (Dubey *et al.*, 1998):

1) **Tachyzoites** in groups or clones

The stage that multiplies rapidly in any cells of the intermediate host and in nonintestinal epithelial cells of the definitive host. Tachyzoites have also been termed endodyozoites or endozoites. They are often crescent shaped, approximately 2 by 6 μm, with a pointed anterior (conoidal) end and a rounded posterior end.

2) **Bradyzoites** in tissue cysts

The stage that multiplies slowly within a tissue cyst. Bradyzoites have also been called cystozoites. Tissue cysts containing bradyzoites vary in size from 12 μm or less in diameter up to 100 μm or more.

3) **Sporozoites** in oocysts

The unsporulated oocysts are subspherical to spherical and are 10 by 12 μm in diameter while sporulated oocysts are subspherical to ellipsoidal and are 11 by 13 μm in diameter. Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature.

Upon ingestion of tissue cysts, the cat's digestive enzymes lyse tissue cyst wall and the bradyzoites are released. Bradyzoites then penetrate the epithelial cells of small intestine where they undergo initial asexual multiplication. This is followed by the sexual cycle in which male and female gametes migrate into the gut and combine to form a zygote that develops into an immature oocyst. The oocyst, after being shed in the feces, will mature into an extraordinarily resistant entity containing 8 sporozoites. Within 5 days the shed oocysts may sporulate and become infectious to other animals and humans. Sporulated oocysts are highly resistant to environmental conditions and can survive in moist shaded soil or sand for many months.

After ingestion of oocysts by other animals, *T. gondii* may enter the blood and lymphatic systems where it multiplies asexually to form the rapidly dividing tachyzoite. Tachyzoite can invade virtually any cell in any tissue and continue to replicate in this life-stage. As the host's immune response rises to the challenge, the parasites encyst and differentiate to the very slowly dividing bradyzoites. Tissue cysts have a greater predilection for the central nervous system but are also found in heart and skeletal muscle. They may persist in infected host for life. However, ingestion of infected tissues by another mammal may result in infection being passed on by a mechanism similar to that of ingestion of oocysts.

2.1.2 Routes of Transmission

Several routes of transmission are possible. *T. gondii* can be acquired by (1) ingestion of materials contaminated with infectious oocysts, (2) ingestion of tissue cysts contained in raw or undercooked meat from another intermediate host or (3) congenital transmission which occurs when a pregnant woman becomes infected and transmits the parasite to her fetus (Kasper and Boothroyd, 1993).

2.2 Toxoplasmosis

Toxoplasmosis, a ubiquitous protozoan infection caused by *T. gondii*, was first observed in animals in 1908, and in humans in 1923. It exists all over the world and affects roughly 30% of the world's human population though the prevalence varies markedly from country to country (Jackson and Hutchison, 1989). About 40% of US adults has serologic evidence of subclinical infection with *T. gondii.* In France and Germany, about 80% of the general population has serologic evidence of subclinical *T. gondii* infection (Sciammarella, 2001). In the past decade, *T. gondii* has received increased attention as a common but important opportunistic pathogen that affects up to 25% of AIDS patients. Many studies have been focused on how to improve diagnostic testing for toxoplasmosis and to develop subunit vaccines that can prevent or attenuate the disease.

2.2.1 Influences and Symptoms

Most cases of toxoplasmosis in immunocompetent hosts are subclinical or benign. However, severe complications may occur in two target groups listed below:

1) **Pregnant women**

Women who contract *T. gondii* during pregnancy are most likely to pass it on to their unborn children through the placenta during later months of pregnancy (second and third trimester). If a fetus is infected with *T. gondii* during the early months of pregnancy (first trimester), the resulting complications are usually considerably more severe. Complications from infection of a fetus (in utero infection) include miscarriage or stillbirth. Some newborns are obviously ill with toxoplasmosis at birth. These babies may already have evidence of severe eye infections as well as a swollen liver or spleen, jaundice or pneumonia. Some of these babies die within days; those survive may have severe visual impairment, delayed development, mental retardation or seizures. Some babies seem healthy at birth but later reveal evidence of in utero infection, including hearing impairment, fluid accumulation in the brain (hydrocephalus), seizures, learning disabilities or mental retardation (Wong and Remington, 1994).

2) **Immunosuppressed patients**

For immunosuppressed (e.g. organ transplant recipients and patients undergoing chemotherapy) or immunodeficient (AIDS) patients, toxoplasmosis is life threatening. Toxoplasmic encephalitis is one of the major opportunistic

infections of the central nervous system and the most frequent cause of focal brain lesions in patients. Symptoms can include severe headache, stiff neck, fever, delirium, muscle weakness, disturbed vision, clumsiness, speech impairment or seizures. Severe toxoplasmic pneumonia may also develop, with fever, shortness of breath and cough. Some patients have infection in the retina of the eye; others experience heart failure, irregularities of the heart's rhythm and rate, or swelling and inflammation of the sac surrounding the heart (Frenkel, 1988).

2.2.2 Treatment of Toxoplasmosis

2.2.2.1 Antitoxoplasma Drugs

Only a few drugs are frequently used in toxoplasmosis treatment. These drugs are suggested to specifically inhibit the metabolisms of *T. gondii* such as nucleic acid metabolism, protein synthesis or electron transfer system. However, none of them can completely eliminate *T. gondii* in the host and some of them may cause severe side effects in patients (Gross and Pohl, 1996). Four well-established antitoxoplasma drugs are introduced below:

1) **Pyrimethamine**

Pyrimethamine affects nucleic acid metabolism by inhibiting dihydrofolate reductase in the conversion of hydrofolate to tetrahydrofolate. This reaction

occurs in both the host and parasite cells. Since dihydrofolate reductase of *T. gondii* possesses a significant higher affinity for pyrimethamine than that of the host cells, toxicity for *T. gondii* is more than 1000 times greater than that for the host cells. The combination of pyrimethamine with sulfadiazine, which acts synergistically, is currently the most effective therapy against tachyzoite stage of *T. gondii*.

2) **Sulfadiazine**

Sulfadiazine inhibits nucleic acid synthesis through inhibiting de novo folic acid synthesis in a competitive manner. It has been shown that sulfadiazine induces more severe side effects (such as hypersensitivity) than any other drugs currently used for toxoplasmosis.

3) **Clindamycin**

Through binding to ribosomes, clindamycin inhibits protein synthesis of *T. gondii* and results in reduction of the level of *T. gondii* replication. In the combination with pyrimethamine, clindamycin has received more attention in treatment of cerebral toxoplasmosis in immunosuppressed patients.

4) **Spiramycin**

Like clindamycin, spiramycin inhibits protein synthesis of *T. gondii*. With its exceptional persistence in the placenta, spiramycin is used in treatment of

women in their first months of pregnancy to prevent the spread of infection to their fetuses.

2.2.2.2 *Toxoplasma* Vaccines

Antitoxoplasma therapy can only target the multiplying and metabolically active form of *T. gondii* but not the non-multiplying or metabolically inactive one. With the intense and continuous use of antitoxoplasma drugs, parasite strains resistant to these drugs emerge, making treatment of toxoplasmosis even more difficult. Accompanying with severe side effects like antitoxoplasma agents, chemotherapy is not a satisfactory approach in fighting against *T. gondii* either. The emergence of *T. gondii* as a major opportunistic organism in immunocompromised individuals and the steady increase in economic losses due to animal toxoplasmosis have fuelled the interest in *T. gondii* vaccine development. Mutant strains of *T. gondii* have been employed in farming industry as the first commercial vaccine (Buxton, 1993).

a) Mutant strains of *T. gondii* as vaccine

In veterinary industry, toxoplasmosis is a major cause of economic loss through neonatal mortality, particularly in sheep, goats and pigs. The objectives of vaccination of farm animals are (1) to reduce abortions resulting from transplacental infection of fetuses and (2) to reduce the risk of human exposure resulting from ingestion of infected meat. To fulfill these purposes,

non-persistent strains of *T. gondii* are vaccine candidates. Several mutant strains of *T. gondii* were identified and promising results have been obtained in farming industry.

1) **Temperature-sensitive mutant TS-4**

TS-4 is a temperature-sensitive mutant tachyzoite. Because of its inability to live and divide at 37°C, it does not colonize the host's tissues nor form tissue cysts. McLeod *et al.* (1988) found that intraintestinal immunization of mice with mutant TS-4 was effective in reducing the incidence of congenital transmission. However, immunization does not prevent the formation of cysts by a challenge infection.

2) **S48 mutant**

S48 mutant is an incomplete tachyzoite with losing ability to develop tissue cysts. It is the first commercial vaccine used in sheep (Buxton, 1993). After subcutaneous injection into sheep, S48 mutant multiplies in local drainage lymph node and causes a febrile response between days 5 and 10. During this period, tachyzoites present in blood and antibody can be detected around day 11. Protection from one S48 mutant infection can last for 18 months. However, the problem of transmission cannot be solved and cyst burden seems to be unchanged in newborns of vaccinated animals.

3) **T-263 mutant**

T-263 is a bradyzoite mutant that undergoes partial development in animal intestine and cannot produce oocysts. Therefore, it can prevent disease in cats and minimize oocyst shedding (Dubey, 1996). However, bradyzoites do not survive for more than a few hours at room temperature, so vaccine should be kept frozen throughout the manufacturing process.

b) Subunit vaccine

Due to the great risk of reversion to virulent form, vaccination with live, attenuated organisms in human is impractical. Development of subunit vaccines is thus an alternative way to achieve effective protection of humans against congenital infection and to prevent infection of immunosuppressed individuals. Antibodies in serum of infected hosts recognize a number of *T. gondii* tachyzoite antigens. Some of these antigens can be recognized by a specific class of immunoglobulins which signifying their potential use as subunit vaccines.

Crude extract of *T. gondii* has been used to immunize mice in many studies. Bourguin *et al.* (1993) demonstrated oral immunization with *T. gondii* sonicated in association with cholera toxin could protect mice from subsequent lethal oral infection. In addition, crude *Toxoplasma* extract could protect rats from

congenital toxoplasmosis (Zenner *et al.*, 1999). However, the undefined composition of crude *Toxoplasma* extract made the results complicated and inconsistent, so preliminary fractionation of crude extract had been carried out. Partially purified antigens were fractionated by SDS-PAGE and tested by different immunoassays. A cytoplasmic antigen isolated by affinity chromatography with monoclonal antibody protected mice against lethal challenge with *T. gondii* (Sharma *et al.*, 1984). Protective immunity was mediated by specific CD4+ T cell (Brinkmann *et al.*, 1993). In another studies by Lunden *et al.* (1993), *T. gondii* major antigens (including P30, P22 and a glycoprotein with molecular weight of 6 kDa) incorporated into immunostimulating complexes (iscom) were used to immunize mice and the results showed that antigens in iscom could induce protective immune responses in mice against lethal interperitoneal and oral challenges.

As antigens can induce protective immunity in the host, different methods were used to purify them as subunit vaccines. With the advances in molecular genetics, many surface and excreted/secreted antigen genes of *T. gondii* had been cloned and used in vaccine development.

2.3 Major *T. gondii* Surface Antigen – P30

P30, also referred to as SAG1 (surface antigen 1), is now recognized as a major surface antigen of *T. gondii*. It is a stage-specific antigen since it can be detected only in rapidly dividing and invasive tachyzoites and neither in bradyzoites of tissue cysts nor sporozoites from oocysts (Kasper *et al.*, 1984).

Biochemical analysis reveals that P30 has an apparent molecular weight of 30 kDa under reducing conditions and approximately 27 kDa under non-reducing conditions (Kasper *et al.*, 1983). As determined by radioimmunoassay, P30 is very abundant. It accounts for about 5% of the total tachyzoite protein (Santoro *et al.*, 1985). P30 is conserved in most strains (Ware and Kasper, 1987) and present both in vesicular network of the parasitophorous vacuole and on the surface of the parasite (Sibley *et al.*, 1986).

P30 is highly immunogenic and can stimulate organism to elicit IgG, IgM, IgA antibodies (Partanen *et al.*, 1984) and interferon γ (Khan *et al.*, 1988). Purified P30 has been tested as a single antigen for serodiagnosis of acute and chronic toxoplasmosis. Protective role of P30 was first suggested by passive transfer of anti-P30 monoclonal antibody into mice (Johnson *et al.*, 1983). Active immunization of P30 incorporated into liposomes also led to a nearly total protection of infected mice (Bulow and Boothroyd, 1991). These results indicate that P30 is

an appropriate antigen for development into a subunit vaccine for immunization of humans and domestic livestock.

Besides its important role in immune mechanism, P30 also plays an important functional role in invasion. Both monoclonal and polyclonal monospecific P30 antibodies inhibit infection of human fibroblasts and murine enterocytes. Fab fragments prepared from polyclonal, monospecific antibody to P30 also have inhibitory effect on invasion, indicating that this antibody directly blocks *T. gondii* infection of the host cells rather than agglutinating it. It is likely that there is a glycosylated host cell receptor to which P30 can bind (Kasper and Khan, 1993).

The gene encoding P30 has been cloned and completely sequenced (Burg *et al.*, 1988). It contains no introns. Hybridization studies indicate that P30 is a single copy gene with mRNA of 1,500 nucleotides in length. Primary sequence of P30 contains processing signals in its translated product. Firstly, it contains an NH_2-terminal signal sequence for entry into lumen of endoplasmic reticulum (ER) and secondly, it contains a glycosyl-phosphatidylinositol (GPI) signal sequence at COOH-terminal (Tomavo, 1996). One putative N-linked glycosylation site (Asn-Xxx-Ser/Thr) appears but is not used in *T. gondii* (Boothroyd *et al.*, 1998).

With all the current understanding of P30, recombinant P30 protein had been produced in different expression systems. A fusion protein containing the mature

part of P30 supplied with six histidyl residues in the N-terminal end was expressed in *Escherichia coli* by Harning *et al.* (1996). Recombinant protein purified on a Ni-chelate column could be recognized by *T. gondii*-specific human IgG and IgM antibodies. Chen *et al.* (1991) expressed His-tagged P30 in an insect cell culture infected with recombinant baculovirus. Mice injected with recombinant protein produced specific humoral and cellular immune responses against *Toxoplasma* infection. Conformationally appropriate expression of P30 was also performed in CHO cells (Kim *et al.*, 1994). Human sera reactive against *Toxoplasma* proteins could recognize purified recombinant P30 protein. Besides, P30 lacking GPI anchor was produced in the methylotrophic yeast, *Pichia pastoris* (Biemans *et al.*, 1998). Despite incomplete processing and unnatural glycosylation, anchor-less P30 protein could be recognized by P30 specific monoclonal and human serum-derived antibodies and protected mice against a lethal challenge of *T. gondii* tachyzoites. Until now, many reports on P30 expression have been published, however, none of them is on plant expression systems.

2.4 Plants as Bioreactors

During the past decade, significant advances have taken place in the field of plant molecular biology and plant biotechnology has also gained increasing scientific and economic importance all over the world. Many plant species can now be transformed through different methods and are even capable of producing very complex proteins such as antibodies and vaccines. A major commercial interest is currently focusing on the ability of transgenic plants to produce recombinant proteins in bulk amounts at relatively low cost, that is, to utilize plants as bioreactors.

2.4.1 Advantages of Plant Bioreactors

Using plant expression systems as bioreactors is attractive because it offers noteworthy advantages over other expression systems based on bacterial, microbial or animal cells. Firstly, the production and scale-up cost for plant expression system is lower than those for other systems. Due to plants' photoautotrophic nature, plant-based production requires only transgenic plants, sunlight, air, land, water, and fertilizers but not the dedicated equipment and expensive medium supplements. With modern agricultural practice, scale-up, harvesting, transporting, storage and processing of plants become easy and efficient (Fischer *et al.*, 1999). Plant systems also take advantages in expressing eukaryotic genes, as nearly all of

the post-translational modifications essential for optimal biological activity of proteins are available. Furthermore, contamination of expressed proteins with human or animal pathogens or other infectious diseases will not occur in plants (Fischer and Emans, 2000). Stable integration of foreign genes into plant chromosomes and subsequent clonal expansion of transgenic plants can pass on the integrated foreign genes from generation to generation in a Mendelian fashion and if desired, homozygotes with increased levels of foreign gene expression can be selected (Arakawa *et al.*, 1999).

2.4.2 Plant-based Vaccines

Vaccination has dramatically improved the quality of human health. Although vaccines are the most cost effective form of health care, however, their worldwide distribution is hampered, especially in developing countries. As plants can be engineered as novel biomanufacturing systems, it is possible to use plants as a source of "edible vaccines". This kind of vaccines is attractive as no cold storage or sophisticated expertise for their distribution is required (Fischer and Emans, 2000). Many studies have been carried out, demonstrating the potential utility of plant-derived subunit vaccine as the basis for vaccine development.

2.4.2.1 VP2 Capsid Protein of Mink Enteritis Virus

Mink enteritis virus (MEV) is an autonomous paravovirus that infects mink. Its major clinical manifestation is a severe inflammation of intestine, resulting in profuse diarrhea. Infection of MEV can be controlled by vaccination.

DNA sequence coding for a short linear epitope from the VP2 capsid protein of MEV was inserted into an infectious cDNA clone of cowpea mosaic virus (CMV). The construct was propagated in the black-eyed bean, *Vigna unguiculata*. As demonstrated by Dalsgaard *et al.* (1997), MEV epitope expressing on the surface of recombinant CMV conferred protection after subcutaneous injection in mink.

2.4.2.2 Hepatitis B Surface Antigen

Hepatitis B virus (HBV) infection is a major health problem in many developing countries. The discovery of surface antigen (HBsAg) enabled the development of HBV vaccine.

In 1992, Mason *et al.* transformed tobacco with the gene encoding HBsAg linked to a nominally constitutive cauliflower mosaic viral (CaMV) 35S promoter. Enzyme-linked immunoassays using a monoclonal antibody directed against human serum-derived HBsAg revealed the presence of recombinant HBsAg in extracts of transformed leaves. Spherical particles with an average diameter of 22 nm were observed in recombinant HBsAg purified from transgenic plants. Sedimentation of

transgenic plant extracts showed that recombinant HBsAg and human serum-derived HBsAg had similar physical properties. As recombinant HBsAg produced in transgenic plants is antigenically and physically similar to that derived from human serum and recombinant yeast, which are used as vaccines, Mason *et al.* (1992) concluded that transgenic plants hold promise as low-cost vaccine production systems.

2.4.2.3 Norwalk Virus Capsid Protein

The capsid protein of Norwalk virus, a calicivirus causing epidemic acute gastroenteritis in humans, was expressed in transgenic tobacco and potato. Under the control of CaMV 35S promoter, recombinant Norwalk virus capsid protein (rNVCP) expression was up to 0.23% of total soluble protein in tobacco leaves. Mice treated with tobacco leaf extracts containing rNVCP developed both serum IgG and secretory IgA specific for rNVCP. Expression of rNVCP driven by paptatin (tuber-specific) promoter was about 0.37% of total soluble protein in potato tuber. When feeding mice directly with transgenic potato tubers, serum IgG specific for rNVCP was induced. These results also indicate the potential use of plants for production and delivery of edible vaccines (Mason *et al.*, 1996).

2.5 Tobacco Expression System

Tobacco, a leafy dicotyledonous plant with a fibrous root system, has served as a most useful plant material in pioneering research with special relevance to plant physiology and biochemistry. It belongs to the Solanaceae family, also known as the potato or nightshade family, and to the genus *Nicotiana*, which was established by Linnaeus in 1753 (Tso, 1972).

Tobacco is one of the most widely adopted model plant used in transgenic experiments primarily because it is readily transformed and subsequently regenerated into whole, fertile plants. Tobacco is normally growing as annual crop and is capable of self-fertilization to produce abundant seeds for analysis.

2.5.1 Transformation Methods

Tobacco plants can be transformed by different methods including *Agrobacterium*-mediated transformation and direct DNA uptake.

2.5.1.1 *Agrobacterium*-mediated Transformation

The soil microorganism *Agrobacterium* is capable of transferring a discrete sequence of DNA called T-DNA from its tumor-inducing (Ti) plasmid into the genome of a broad range of gymnosperms and angiosperms. This natural ability of *Agrobacterium* to transform plants is exploited in the *Agrobacterium*-mediated

transformation method.

Horsch *et al.* (1985) developed a very simple leaf disk method for plant transformation. Basically, it involves immersion of leaf disks in a liquid culture of *Agrobacterium* carrying the chosen transformation vector; cocultivation of leaf disks with *Agrobacterium*; and the transfer of infected leaf disks to suitable medium for plant regeneration.

2.5.1.2 Direct DNA Uptake

Despite the fact that the host range of *Agrobacterium* is broad, a limitation to its use is that some crop species are recalcitrant to this mode of gene transfer. So more "direct DNA transfer" methods have been developed to tackle this problem. Direct DNA uptake is a physical process that does not require any biological vector, thus eliminating any possible host range problem mentioned above.

Biolistic gene transfer (particle bombardment) involves the delivery of microprojectiles, usually of tungsten or gold coated with DNA, into plant cells by high-velocity acceleration. Both transient β-glucuronidase (GUS) expression (Seki *et al.*, 1991a) and stable transformation (Seki *et al.*, 1991b) of *Arabidopsis* demonstrated that it is feasible to transform plants via particle bombardment.

Of different transformation systems, plant protoplast transformation is probably the one that necessitates the most finesse. Protoplasts are isolated either by a

mechanical or by an enzymatic process to remove the cell wall. Polyethylene glycol (PEG) is commonly used to facilitate DNA uptake into protoplasts of both dicots and monocots. In 1987, Negrutiu *et al.* established the protocol of PEG-mediated direct DNA uptake for tobacco. Electroporation can also be used to transform protoplasts. Pores generated on protoplast cell membrane through short electrical pulses of high electric field allow the entry of DNA into protoplasts electrophoretically (Jones, 1995).

2.6 Phaseolin and Its Regulatory Sequences

Phaseolin is the predominant seed storage glycoprotein of French bean (*Phaseolus vulgaris* L.), constituting about 50% of the total protein in mature seeds. It is a globulin consisting of 3 subunit polypeptides, α, β and γ, with apparent molecular weights of 53, 47 and 43 kDa, respectively (Sun *et al.*, 1981). The 3 polypeptides are encoded by 16s mRNA species which are translated on polysomes attached to ER before entering the lumen of ER. The polypeptides are then glycosylated, folded into proper tertiary structure and assembled into a trimer. The trimer transits the Golgi apparatus and is finally deposited in membrane-bound vacuolar protein bodies 1-5 μm in diameter (Hoffman *et al.*, 1988).

Being spatially and temporally regulated, phaseolin is synthesized and accumulated rapidly only in the developing cotyledons, beginning when the cotyledons are about 7 mm in length and continuing until they reach 17-19 mm in length (Sun *et al.*, 1978).

β-phaseolin gene was isolated from French bean by Sun *et al.* in 1981. It contains 80-base pair (bp) 5' untranslated region, 1,263-bp protein-encoding region interrupted by 5 intervening sequences, and 135-bp 3' untranslated region. Since phaseolin is an abundant seed storage protein, representing 50% of the total protein in mature French bean, phaseolin promoter is a good candidate for expressing

recombinant proteins in transgenic plants. Under the control of phaseolin promoter, a chimeric gene encoding the Brazil nut methionine-rich protein was transformed into tobacco (Altenbach *et al.*, 1989) and a significant increase (up to 30%) in methionine content of the transgenic tobacco seeds was observed.

Chapter 3 Expression of P30 in Transgenic Tobacco

3.1 Introduction

Chimeric genes encoding the major surface antigen P30 of *T. gondii* under the control of either constitutive or seed-specific promoter were constructed and transformed into tobacco. All the coding sequences contain a hexa-histidine tag (His-tag) and an enterokinase (EK) recognition site at the 5'end of the P30 gene for recombinant protein detection and purification.

The P30 protein is expected to appear in all parts of the transgenic plants harboring the chimeric gene driven either by the CaMV 35S or the maize ubiquitin 1 (Ubi) constitutive promoter. Transgenic plants containing the P30 gene under the regulation of seed-specific phaseolin promoter are expected to have their P30 protein present in the seed cytosol while those with the phaseolin signal peptide (Phas SP) would have their P30 accumulated in protein bodies.

3.2 Materials and Methods

3.2.1 Chemicals

Most chemicals used were of reagent grade or molecular grade and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) or otherwise noted. Restriction enzymes and other modifying enzymes for molecular biology experiments were obtained from New England Biolabs Inc. (Beverly, MA, USA) and Promega Biosciences (Madison, WI, USA) unless specified.

3.2.2 Oligos: Primers and Adapters

Oligos listed in Table 3- 1 were supplied by Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Adapters were prepared by mixing 100 pmole of each concerning oligo and allowing them to anneal as follows: 88°C for 2 minutes, 65°C for 10 minutes, 37°C for 10 minutes and then 25°C for 5 minutes. Phosphorylation of the annealed adapters was performed with 1 mM ATP and 10 units of T4 polynucleotide kinase (Promega) in 1X T4 polynucleotide kinase buffer at 37°C for 30 minutes.

Table 3- 1 Sequences of oligos used in this study

Name of oligo DNA	Sequence (5' to 3')	Purpose
P30-5'	CCCACTCTTGCGTACTACCCCA	PCR screening of P30 clones
P30-3'	CTTGATCGTTAGCGTGGCACCC	PCR screening of P30 clones; Synthesis of DIG-labeled DNA Probe
5'P30-PCR	TCTAGAATTCATGCACCATCATCATCATCATGAC	PCR screening of P30 clones
3'P30-PCR	ATACAAGCTTTCACCCGGCAAACTCCAGTTTC	PCR screening of P30 clones
P30-5'mid	GTGACAGTACAAGCCAGAGCCT	Sequencing
P30-3'mid	GAGTGCTGTCTGCACCGTAGG	Sequencing
P30-1D	TATGCCACTTCACTCCACCATCATCATCATCATCCA	Generation of P30-D adapter
P30-2D	GATCTGGATGATGATGATGATGGTGGAGTGAAGTGG CA	Generation of P30-D adapter
P30-1E'	TATGGTACCGTCTACATGCACCATCATCATCATCAT GACGACGACGACAAGGC	Generation of P30-E adapter
P30-2E'	CATGGCCTTGTCGTCGTCGTCATGATGATGATGATG GTGCATGTAGACGGTACCA	Generation of P30-E adapter
P30-1F	GGGTGAGTATACGGATCCA	Generation of P30-F adapter
P30-2F	AGCTTGGATCCGTATACTCACCCTGCA	Generation of P30-F adapter

3.2.3 Plant Materials

Seeds of wild type tobacco (*Nicotiana tabacum var. xanthi* N.C.) were surface sterilized in 100% Clorox (5.25% sodium hypochlorite) for 3 minutes with vigorous shaking, followed by washing with sterilized distilled water 3 times, each for 1 minute. Sterilized seeds were allowed to germinate on MS agar [4.3 g/L Murashige & Skoog salts (Gibco), 2% sucrose, 1X vitamin B5 and 0.8% bacto-agar, pH5.7] and grow at 25°C with an 18-hour photoperiod. Tobacco leaves about one-month old were used for *Agrobacterium*-mediated transformation. After transformation, regenerated transgenic tobacco plants were transferred to soil and greenhouse for seed formation and subsequent analysis.

3.2.4 Bacterial Strains

Escherichia coli strain DH5α was used as a host for plasmid manipulation and maintenance. *Agrobacterium tumefaciens* strain LBA4404 containing the helper Ti plasmid pAL4404 was used in tobacco transformation.

3.2.5 Construction of Chimeric Genes

The original P30 coding sequence was isolated by Chen *et al.* (1994) from *T. gondii* strain ZS1 using polymerase chain reaction (PCR) technique. It was then modified by Wong and Lo (Chinese Univ., Hong Kong) to have the 5' signal peptide

(SP) and the 3' glycosyl-phosphatidyl inositol (GPI) signal sequence removed. The modified P30 cDNA was then subcloned into the bacterial expression vector pET30a(+) (Novagen) to form pET-ΔSP30ΔPI.

In this study, the overall strategy for construction of chimeric genes was first to add coding sequences for His-tag and EK recognition site at the 5' end of the P30 gene and to create suitable restriction sites for cloning with the use of adapters (Table 3- 1 & Figure 3- 1). The modified P30 gene was then subcloned into a vector containing either the CaMV 35S, Ubi or phaseolin (with or without SP) promoter. Finally, the resulting cassettes with the P30 gene sandwiched between a promoter and a terminator were inserted into the *Agrobacterium* binary vector pBI121 for tobacco transformation.

3.2.5.1 Modification of pET-ΔSP30ΔPI

In the presence of adapters, pET-ΔSP30ΔPI carrying the P30 cDNA with its SP and GPI anchor removed was first modified to facilitate further cloning.

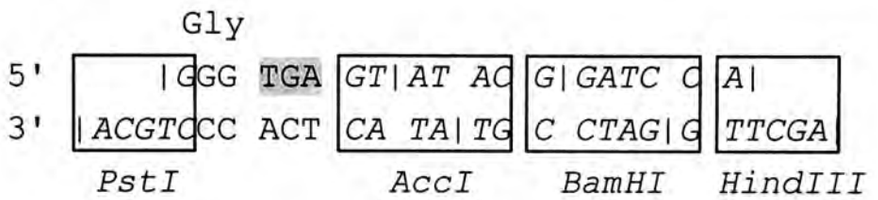
As shown in Figure 3- 2, pET/P30F was constructed by inserting adapter P30-F (Figure 3- 1) in between the PstI and HindIII sites of pET-ΔSP30ΔPI. Through this modification, a stop codon (TGA) and an AccI and BamHI sites were added at the 3' end of the P30 cDNA.

For construction of pET/DP30F (Figure 3- 3), pET/P30F was first cut with NdeI

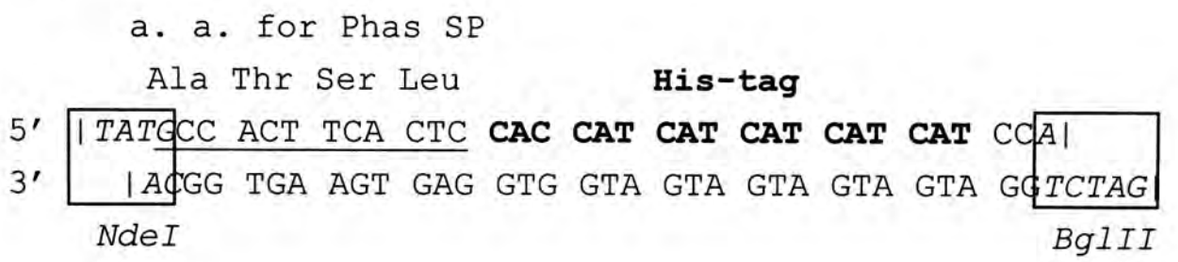
and BglII. With addition of adapter P30-D (Figure 3- 1) in between the NdeI and BglII sites of pET/P30F, pET/DP30F emerged with sequences coding for 4 additional amino acids (Ala, Thr, Ser and Leu) at the end of the phaseolin SP, a His-tag and an EK site at the 5' end of the P30 cDNA. The pET/DP30F was then used to construct of chimeric gene with the phaseolin promoter and phaseolin SP.

With insertion of adapter P30-E' (Figure 3- 1) at the NdeI and NcoI sites of pET/P30F, pET/E'P30F was formed (Figure 3- 4). The pET/E'P30F, with its KpnI, AccI, start codon (ATG), His-tag and EK sites at the 5' end of the P30 cDNA, was used in further cloning including the addition of the CaMV 35S, Ubi or phaseolin promoter.

Adapter P30-F



Adapter P30-D



Adapter P30-E'

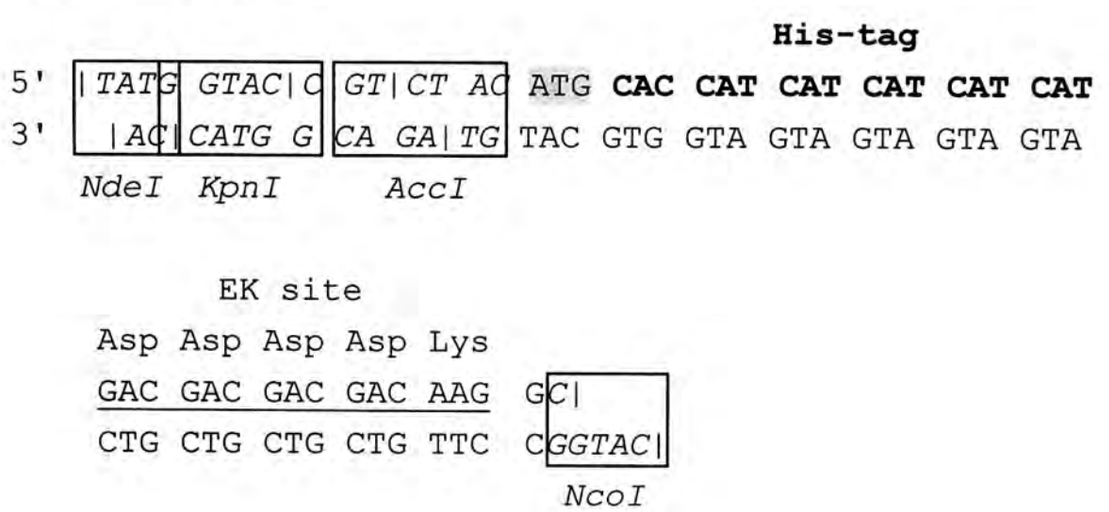


Figure 3- 1 Adapters for modification of pET-ΔSP30ΔPI

Start (ATG) and stop (TGA) codons are shaded. His-tag coding sequence is bolded and the sequences encoding 4 additional amino acids in the phaseolin SP in adapter P30-D and EK site in adapter P30-E' are underlined.

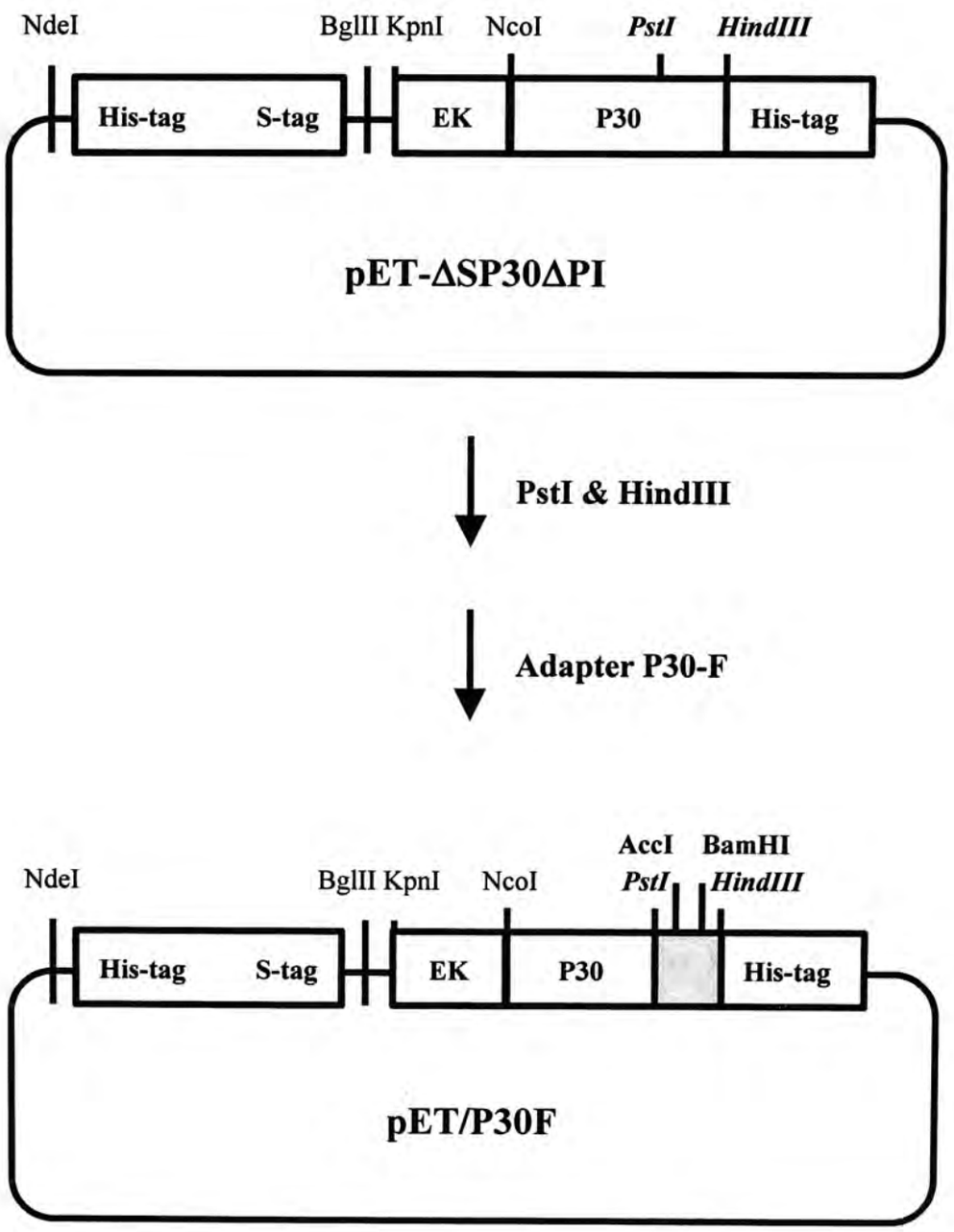


Figure 3- 2 Construction of chimeric gene pET/P30F

Adapter P30-F was inserted in between the PstI and HindIII sites of pET-ΔSP30ΔPI. The resulting construct, pET/P30F, contains a stop codon and an AccI and a BamHI sites at the 3' end of the P30 gene.

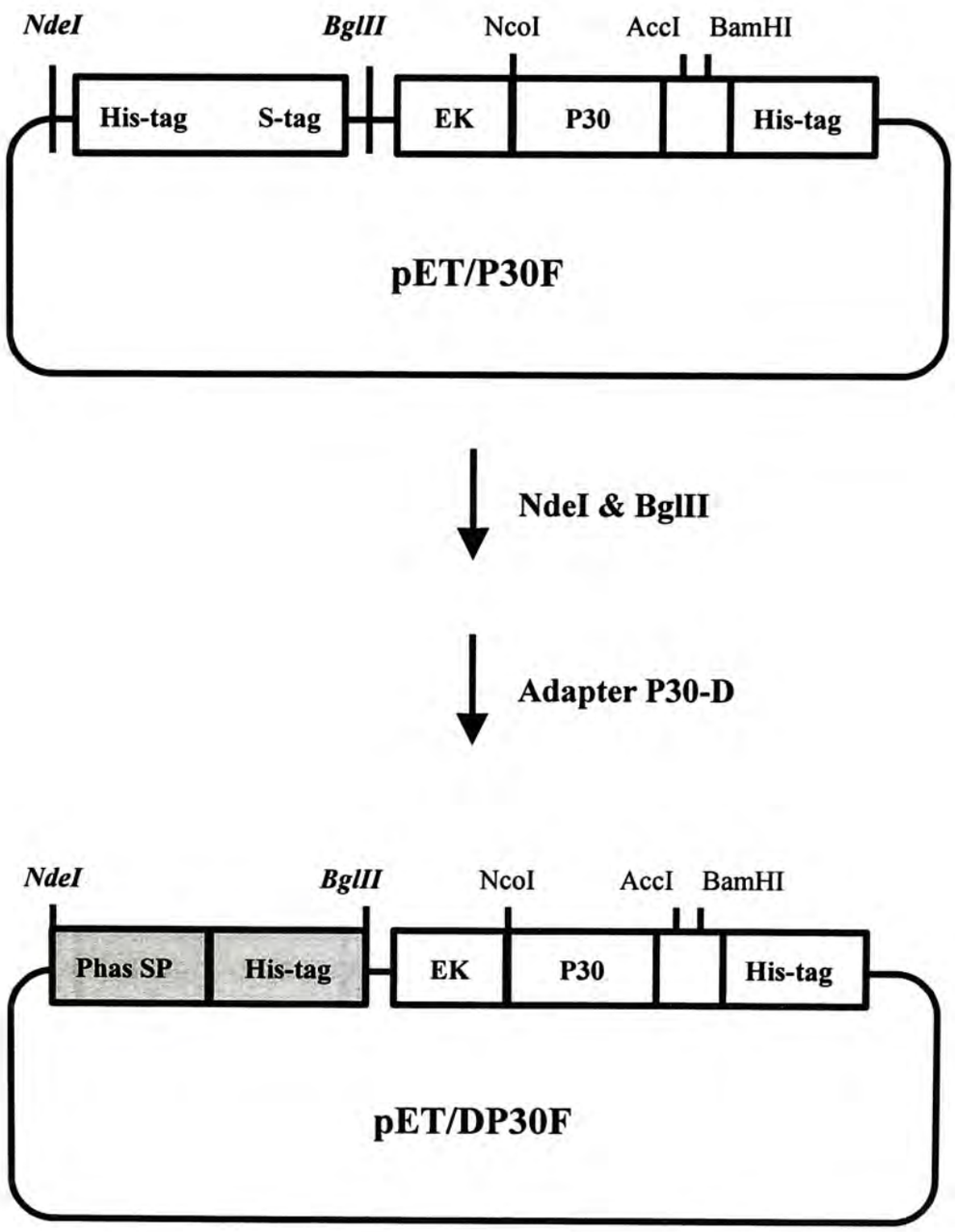


Figure 3- 3 Construction of chimeric gene pET/DP30F

With insertion of adapter P30-D in between NdeI and BglII of pET/P30F, pET/DP30F was constructed with sequences encoding 4 additional amino acids in the phaseolin SP, a His-tag and an EK site at the 5' end of the P30 cDNA.

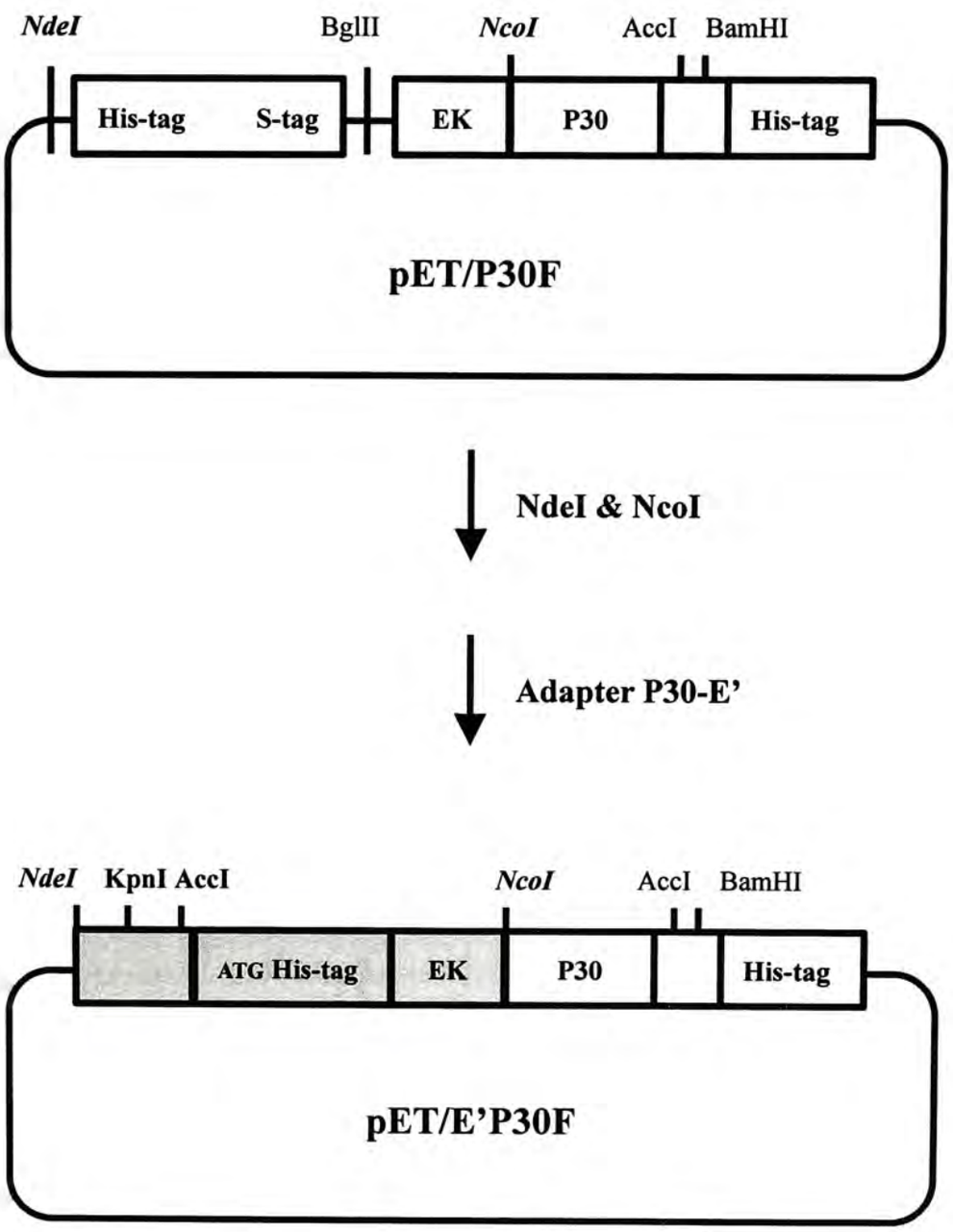


Figure 3- 4 Construction of chimeric gene pET/E'P30F

With addition of adapter P30-E' at the NdeI and NcoI sites of pET/P30F, pET/E'P30F was constructed with sequences encoding a start codon, a His-tag and an EK site at the 5' end of the P30 cDNA.

3.2.5.2 Cloning of P30 into Vectors with Different Promoters

Promoters used in this study can be divided into 2 categories:

a) **Constitutive promoter** – The CaMV 35S & Ubi promoters;

b) **Seed-specific promoter** – The phaseolin promoter with or without phaseolin SP.

3.2.5.2.1 Cloning of P30 into Vector with CaMV 35S Promoter

The P30 gene from pET/E'P30F was subcloned into pUC/35S, a vector with the CaMV 35S promoter (pro) and the nopaline synthase (Nos) terminator (ter), through insertion between the KpnI and BamHI sites as illustrated in Figure 3- 5, resulting in pUC/35S-HP.

3.2.5.2.2 Cloning of P30 into Vector with Maize Ubiquitin 1 Promoter

pUC/Ubi-HP, with its P30 cDNA regulated by the Ubi promoter and Nos terminator, was constructed using the same strategy as pUC/35S-HP (Figure 3- 6).

3.2.5.2.3 Cloning of P30 into Vector with Phaseolin Promoter

As shown in Figure 3- 7, the P30 insert in pET/E'P30F was excised using AccI and subcloned into pTZ/Phas (the vector containing the phaseolin promoter and phaseolin terminator) to form pTZ/Phas-HP.

3.2.5.2.4 Cloning of P30 into Vector with Phaseolin Promoter and Phaseolin SP

The P30 gene was excised from pET/DP30F and subcloned into pBK/Phas.SP between the NdeI and AccI sites. The resulting construct, pBK/Phas.SP-HP, contained the phaseolin promoter and phaseolin SP (Figure 3- 8).

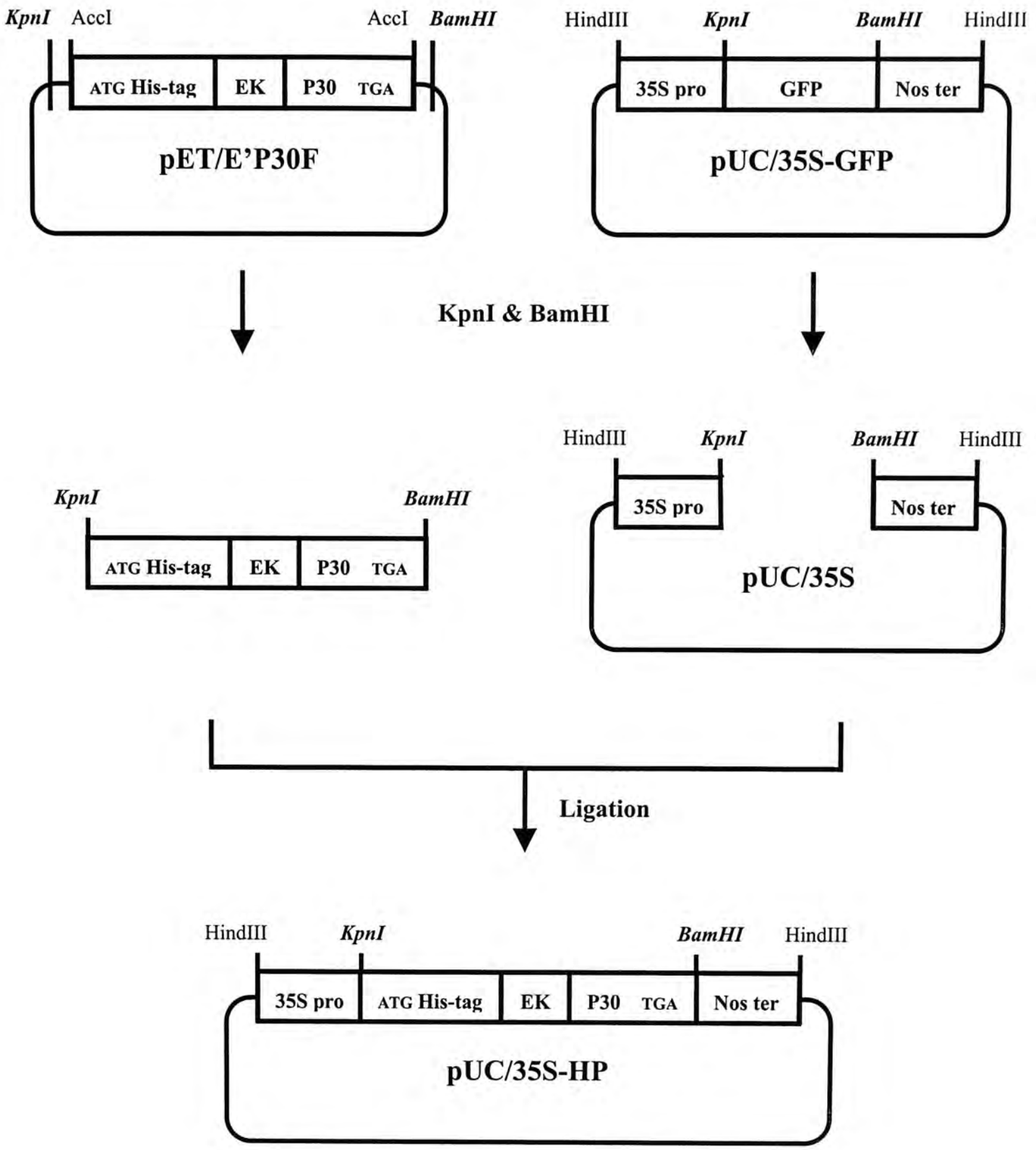


Figure 3- 5 Construction of chimeric gene pUC/35S-HP

The P30 gene from pET/E'P30F was subcloned into pUC/35S-GFP between the KpnI and BamHI sites. The resulting construct, pUC/35S-HP, contained the P30 gene sandwiched between the CaMV 35S promoter and Nos terminator. (GFP = green fluorescent protein)

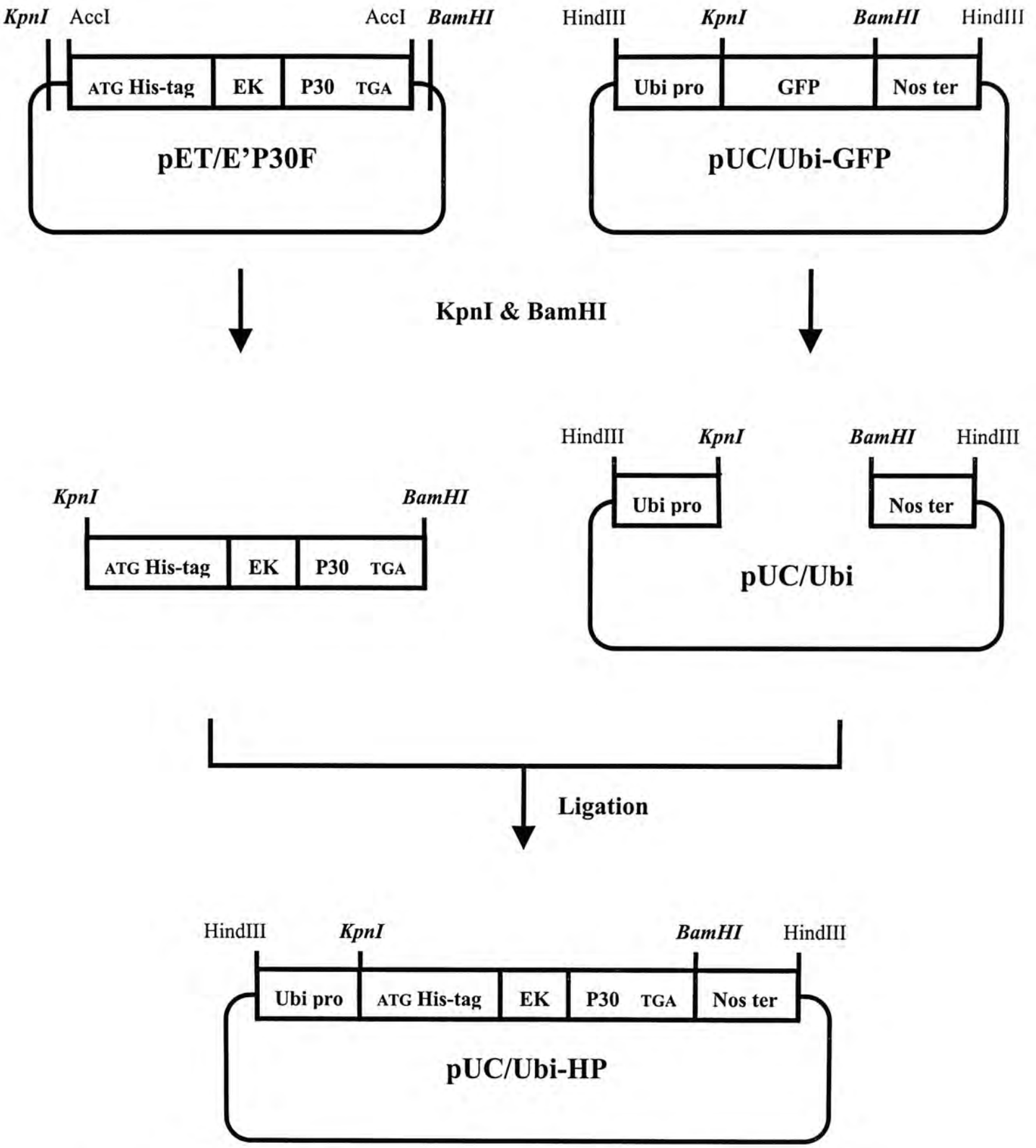


Figure 3- 6 Construction of chimeric gene pUC/Ubi-HP

The P30 gene from pET/E'P30F was subcloned into pUC/Ubi-GFP between the KpnI and BamHI sites. The resulting construct, pUC/Ubi-HP, contained the P30 gene sandwiched between the Ubi promoter and Nos terminator. (GFP = green fluorescent protein)

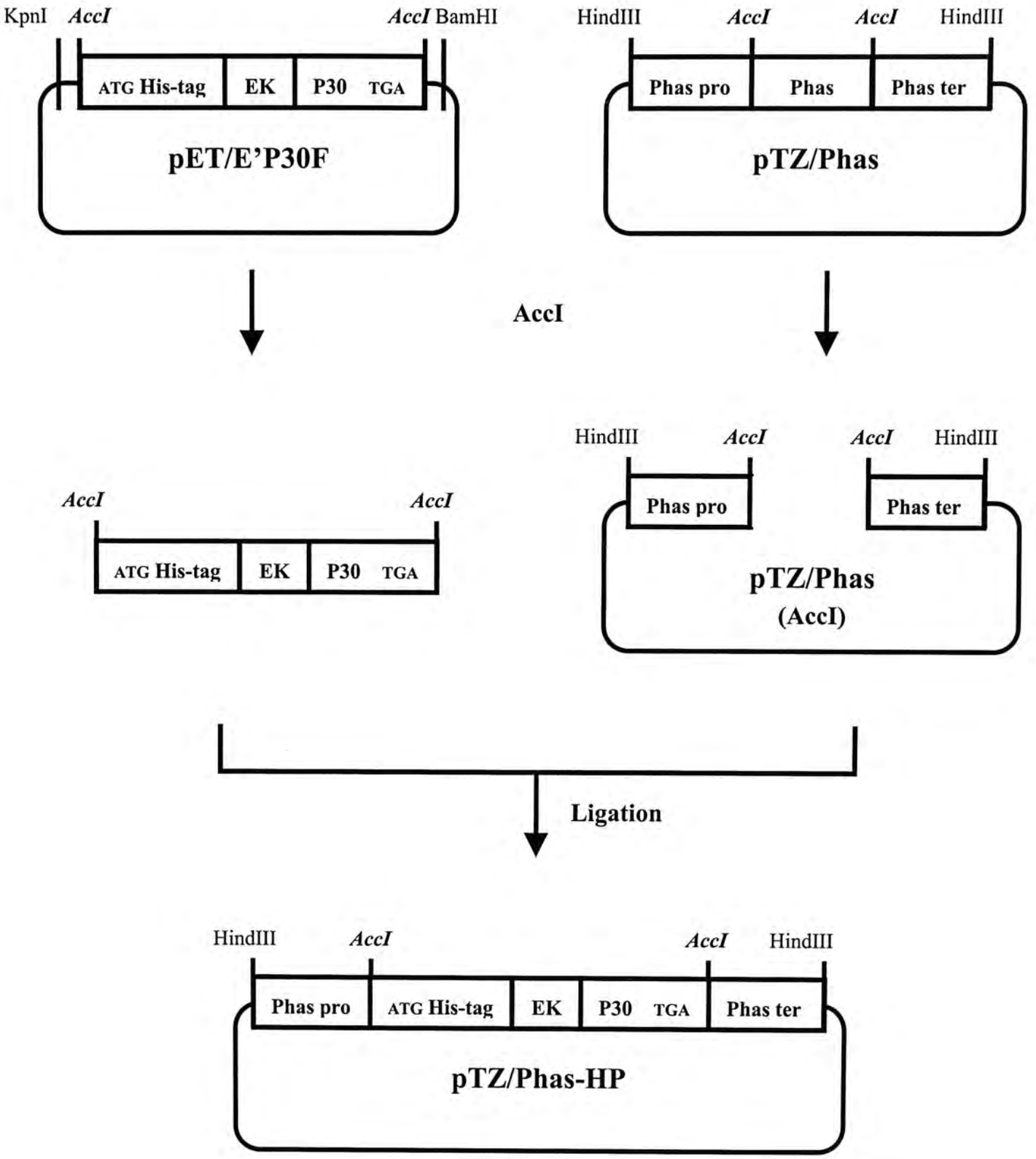


Figure 3- 7 Construction of chimeric gene pTZ/Phas-HP

The P30 insert was excised from pET/E'P30F using AccI and subcloned into pTZ/Phas. The resulting construct, pTZ/Phas-HP, contained the phaseolin (Phas) promoter and terminator flanking the P30 gene.

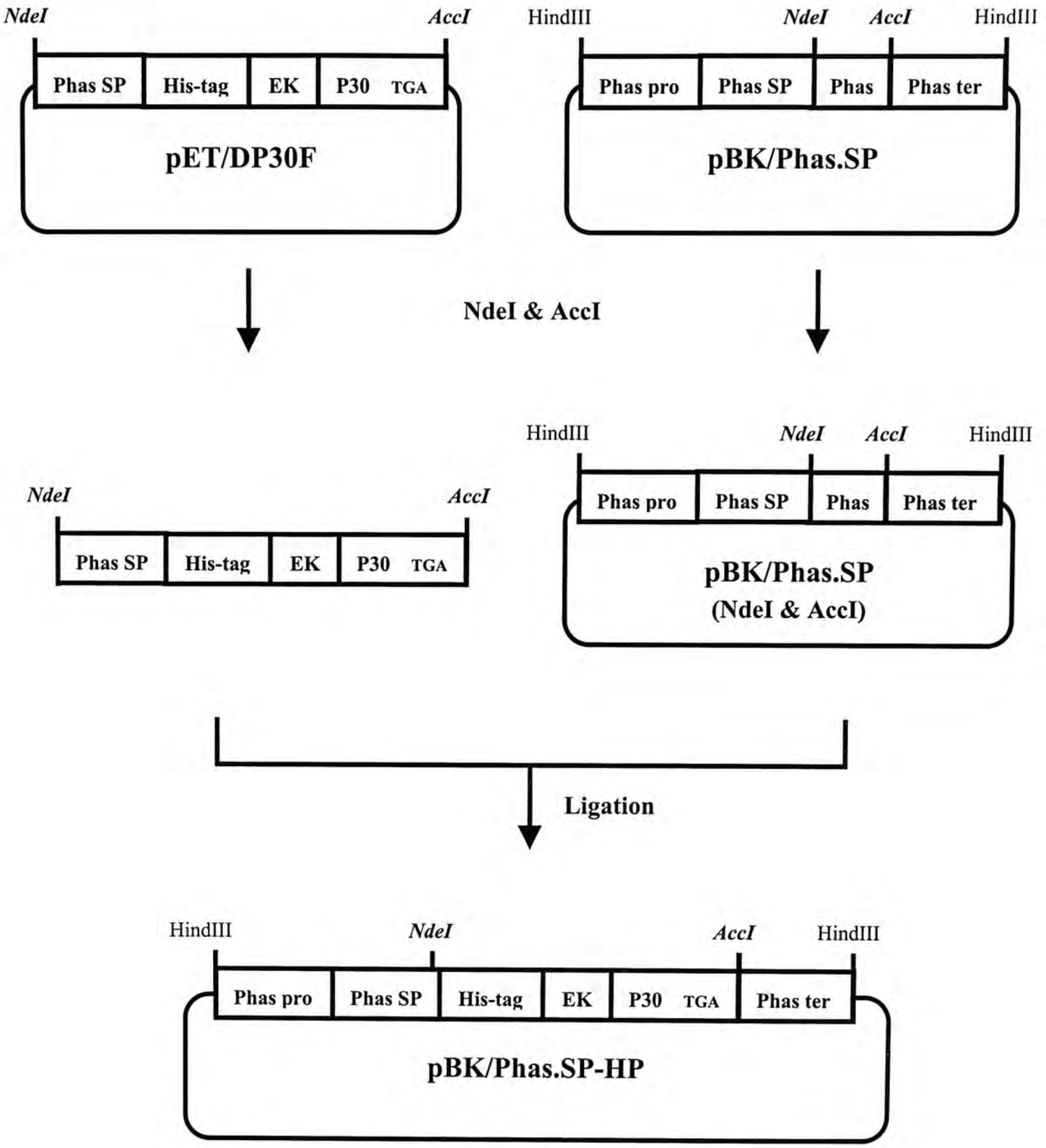


Figure 3- 8 Construction of chimeric gene pBK/Phas.SP-HP

The P30 gene was excised from pET/DP30F and subcloned into pBK/Phas.SP through insertion between the NdeI and AccI sites. The resulting construct, pBK/Phas.SP-HP, contained the P30 gene sandwiched between the phaseolin (Phas) promoter with SP (signal peptide) and terminator.

3.2.5.3 Cloning of P30 into *Agrobacterium* Binary Vector pBI121

Four chimeric P30 constructs, the pUC/35S-HP, pUC/Ubi-HP, pTZ/Phas-HP and pBK/Phas.SP-HP, were cut with HindIII to release the promoter-P30-terminator cassettes. These cassettes were then inserted into the HindIII site of *Agrobacterium* binary vector pBI121 (Clontech). The resulting constructs (Figure 3- 9), containing the promoter-P30-terminator cassette sandwiched between neomycin phosphotransferase II (NPTII) selectable marker and β-glucuronidase (GUS) reporter genes, were transformed into *Agrobacterium tumefaciens* LBA4404 with the helper Ti plasmid pAL4404 by electroporation. The nucleotide and amino acid sequences of P30 in pBI/35S-HP, pBI/Ubi-HP and pBI/Phas-HP were illustrated in Figures 3- 10 and 3- 12 while those of pBI/Phas.SP-HP were shown in Figures 3- 11 and 3- 13.

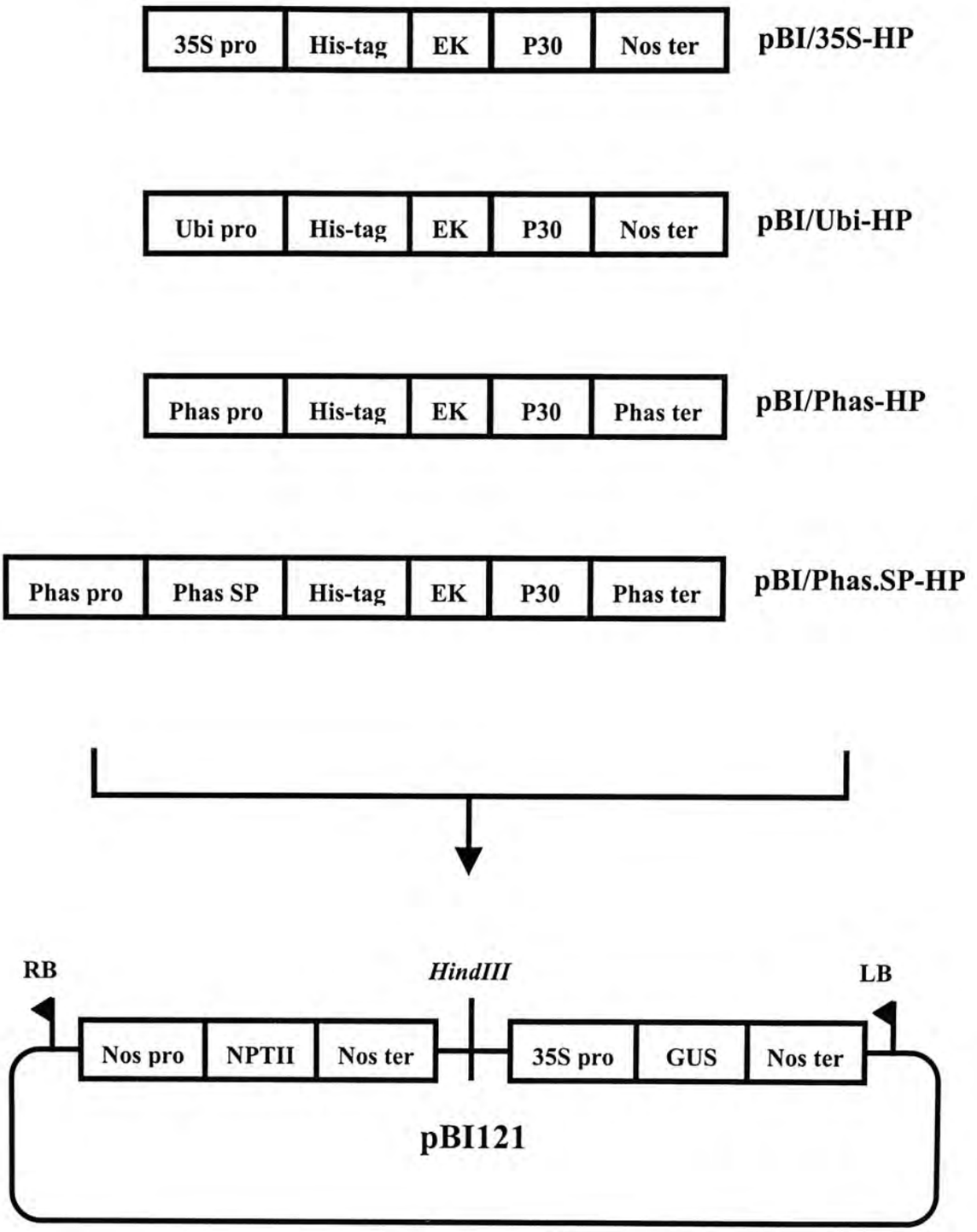


Figure 3- 9 Cloning of chimeric P30 genes into *Agrobacterium* binary vector pBI121

The promoter-P30-terminator cassette was excised from pUC/35S-HP, pUC/Ubi-HP, pTZ/Phas-HP or pBK/Phas.SP-HP using HindIII and inserted into the HindIII site of *Agrobacterium* binary vector pBI121. (RB / LB = right border / left border of T-DNA)

ATG**CACCATCATCATCATCAT**GACGACGACGACAAGGCCATGGGTTTCACTCTTAAG

TGCCCTAAAACAGCGCTCACAGAGCCTCCCACTCTTGCGTACTCACCCAACAGGCAA

ATCTGCCCAGCGGGTACTACAAGTAGCTGTACATCAAAGGCTGTAACATTGAGCTCC

TTGATTCCTGAAGCAGAAGATAGCTGGTGGACGGGGGATTCTGCTAGTCTCGACACG

GCAGGCATCAAACTCACAGTTCCAATCGAGAAGTTCCCCGTGACAACGCAGACGTTT

GTGGTCGGTTGCATCAAGGGAGACGACGCACAGAGTTGTATGGTCACAGTGACAGTA

CAAGCCAGAGCCTCATCGGTCGTCAATAATGTCGCAAGGTGCTCCTACGGTGCAGAC

AGCACTCTTGGTCCTGTCAAGTTGTCTGCGGAAGGACCCACTACAATGACCCTCGTG

TGCGGGAAAGATGGAGTCAAAGTTCCTCAAGACAACAATCAGTACTGTTCCGGGACG

ACGCTGACTGGTTGCAACGAGAAATCGTTCAAAGATATTTTGCCAAAATTAACTGAG

AACCCGTGGCAGGGTAACGCTTCGAGTGATAAGGGTGCCACGCTAACGATCAAGAAG

GAAGCATTTCCAGCCGAGTCAAAAAGCGTCATTATTGGATGCACAGGGGGATCGCCT

GAGAAGCATCACTGTACCGTGAAACTGGAGTTTGCCGGGGCTGCAGGGTGA

Figure 3- 10 Nucleotide sequence of the P30 gene in pBI/35S-HP, pBI/Ubi-HP or pBI/Phas-HP

The length of the P30 gene in pBI/35S-HP, pBI/Ubi-HP or pBI/Phas-HP is 735 bp. The start (ATG) and stop (TGA) codons are shaded. A His-tag coding sequence (bolded) and an EK recognition sequence (underlined) are located at the 5'end of the P30 coding sequence.

ATG*atgagagcaagggttccactcctgttgctgggaattctttcctggcatcactt*

*tctgcctcatatgccacttcactc***CACCATCATCATCATCAT**CCAGATCTGGGTACC

GACGACGACGACAAGGCCATGGGTTTCACTCTTAAGTGCCCTAAAACAGCGCTCACA

GAGCCTCCCACTCTTGCGTACTCACCCAACAGGCAAATCTGCCCAGCGGGTACTACA

AGTAGCTGTACATCAAAGGCTGTAACATTGAGCTCCTTGATTCCTGAAGCAGAAGAT

AGCTGGTGGACGGGGGATTCTGCTAGTCTCGACACGGCAGGCATCAAACTCACAGTT

CCAATCGAGAAGTTCCCCGTGACAACGCAGACGTTTGTGGTCGGTTGCATCAAGGGA

GACGACGCACAGAGTTGTATGGTCACAGTGACAGTACAAGCCAGAGCCTCATCGGTC

GTCAATAATGTCGCAAGGTGCTCCTACGGTGCAGACAGCACTCTTGGTCCTGTCAAG

TTGTCTGCGGAAGGACCCACTACAATGACCCTCGTGTGCGGGAAAGATGGAGTCAAA

GTTCCTCAAGACAACAATCAGTACTGTTCCGGGACGACGCTGACTGGTTGCAACGAG

AAATCGTTCAAAGATATTTTGCCAAAATTAACTGAGAACCCGTGGCAGGGTAACGCT

TCGAGTGATAAGGGTGCCACGCTAACGATCAAGAAGGAAGCATTTCCAGCCGAGTCA

AAAAGCGTCATTATTGGATGCACAGGGGGATCGCCTGAGAAGCATCACTGTACCGTG

AAACTGGAGTTTGCCGGGGCTGCAGGGTGA

Figure 3- 11 Nucleotide sequence of the P30 gene in pBI/Phas.SP-HP

The length of the P30 gene in pBI/Phas.SP-HP is 828 bp. The start (ATG) and stop (TGA) codons are shaded. The sequence of phaseolin SP is in lower case and an arrow indicates the possible SP cleavage site. The His-tag coding sequence is bolded and the EK recognition sequence is underlined.

M**HHHHHH**DDDDKAMGFTLKCPKTALTEPPTLAYSPNRQICPAGTTSSCTSKAVTLSS

LIPEAEDSWWTGDSASLDTAGIKLTVPIEKFPVTTQTFVVGCIKGDDAQSCMVTVTV

QARASSVVNNVARCSYGADSTLGPVKLSAEGPTTMTLVCGKDGVKVPQDNNQYCSGT

TLTGCNEKSFKDILPKLTENPWQGNASSDKGATLTIKKEAFPAESKSVIIGCTGGSP

EKHHCTVKLEFAGAAG

Figure 3- 12 Deduced amino acid sequence of P30 in pBI/35S-HP, pBI/Ubi-HP or pBI/Phas-HP

The P30 protein from pBI/35S-HP, pBI/Ubi-HP or pBI/Phas-HP is predicted to contain 244 amino acids. The His-tag is bolded while the EK recognition site is underlined.

mmrarvpllllgilflaslsasyatsl↓**HHHHHH**PDLGTDDDDKAMGFTLKCPKTALT

EPPTLAYSPNRQICPAGTTSSCTSKAVTLSSLIPEAEDSWWTGDSASLDTAGIKLTV

PIEKFPVTTQTFVVGCIKGDDAQSCMVTVTVQARASSVVNNVARCSYGADSTLGPVK

LSAEGPTTMTLVCGKDGVKVPQDNNQYCSGTTLTGCNEKSFKDILPKLTENPWQGNA

SSDKGATLTIKKEAFPAESKSVIIGCTGGSPEKHHCTVKLEFAGAAG

Figure 3- 13 Deduced amino acid sequence of P30 in pBI/Phas.SP-HP

The P30 protein from pBI/Phas.SP-HP is predicted to contain 275 amino acids before the cleavage of the phaseolin SP (in lower case). An arrow indicates the possible site for SP cleavage. The mature peptide should have 253 amino acids. The His-tag is bolded while the EK recognition site is underlined.

3.2.6 Transformation of *Agrobacterium* by Electroporation

Plasmids, pBI/35S-HP, pBI/Ubi-HP, pBI/Phas-HP, pBI/Phas.SP-HP and pBI121 (the vector-alone control) were transformed into *Agrobacterium tumefaciens* LBA4404 with the helper Ti plasmid pAL4404 by electroporation.

For each construct, an aliquot of 40 μl of *A. tumefaciens* LBA4404 competent cells was thawed on ice, mixed with 10 ng of plasmid DNA and kept on ice for 1 minute. The cell-DNA mix was then transferred to the bottom of a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad) and subjected to a pulse of 25 μF, 2.5 kV and 600 ohms in the Gene Pulser (Bio-Rad). After discharge, one ml of SOC medium [2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose] was immediately added to the cuvette to rescue the cells. The cell suspension was then transferred to a 10 ml polypropylene tube and shaken at 28°C for 2 hours. The recovered culture was spread on LB agar plates supplemented with 50 mg/L kanamycin (Kan) and 25 mg/L streptomycin and allowed to grow at 28°C for 2-3 days.

3.2.7 Transformation, Selection and Regeneration of Tobacco

Transformation of tobacco was carried out as described by Lin *et al.* (1994) with modifications. A single colony of *A. tumefaciens* LBA4404 harboring the pBI121, pBI/Phas-HP, pBI/Phas.SP-HP, pBI/35S-HP or pBI/Ubi-HP was cultured in 3 ml of LB medium [10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 10 g/L NaCl] containing 50 mg/L Kan and 25 mg/L streptomycin at 30°C with shaking for 16 hours. This 3-ml starter culture was then subcultured in 300 ml of YM broth [0.4 g/L autolyzed, low sodium yeast extract, 10 g/L mannitol, 0.1 g/L NaCl, 0.5 g/L K_2HPO_4 and 0.2 g/L $MgSO_4 \bullet 7H_2O$] supplemented with 50 mg/L Kan and 25 mg/L streptomycin and allowed to grow at 30°C with shaking until its optical density 620 nm (OD_{620}) reached 0.8-1.0 (5 x 10^8 cells/ml for 1 OD_{620}). The cells were centrifuged at 7,000 rpm for 10 minutes and the pellets were resuspended in MS medium with 0.5 mg/L MES to a concentration of 10^{10} cells/ml.

Young tobacco leaves from one-month-old plants were removed and cut into small pieces (about 1 cm x 1 cm square disks). The explants were incubated with *Agrobacterium* cells for 10 minutes, transferred to solid MS medium with lower side facing up and incubated for 2 days at 25°C, using an 18 hour light/ 6 hour dark cycle. After cocultivation, the explants were transferred to shoot regeneration medium [MS agar plus 0.5% N^6-benzyladenine (BA), 500 mg/L carbenicillin (Car) and 100 mg/L

Kan] and incubated at 25°C, using an 18 hour light/ 6 hour dark cycle until the formation of green calli and shoots. For root regeneration, the regenerated shoots were transferred to MS agar containing 500 mg/L Car and 100 mg/L Kan in Magenta boxes. Transgenic tobacco plants were finally transferred to soil in pots and allowed to grow in greenhouse.

3.2.8 GUS Assay

GUS assay was performed as described by Jefferson *et al.* (1987). Regenerated tobacco leaves was soaked in GUS staining solution [100 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 1 mM EDTA, 0.5 mM K_3•Fe(CN)$_6$, 0.05 mM K_4•Fe(CN)$_6$ and 0.5 mg/ml X-glucuronide (X-gluc)] at 37°C overnight for color development. Chlorophyll background was removed by soaking in 70% ethanol (EtOH).

3.2.9 Synthesis of Single-stranded DIG-labeled DNA Probe

Anti-sense DIG-labeled P30 DNA probe was synthesized by PCR technique. A P30 fragment, serving as template for PCR, was excised from pET/E'P30F (Figure 3- 4) by KpnI and HindIII double digestion and recovered with the QIAquick Gel Extraction Kit (QIAGEN). A 100 μl reaction mix containing 50 ng of the P30 template, 1X PCR buffer, 1 mM $MgCl_2$, 20 μM DIG dNTPs (Roche), 0.1 μM P30-3' primer (Table 3- 1), and 5 units of Platinum Taq DNA polymerase (Gibco) was

prepared for PCR reaction with the following conditions: 94°C for 10 minutes, 60 cycles of 94°C for 20 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by 1 cycle of 72°C for 10 minutes. The labeling efficiency of the probe was estimated by a spot test using DIG-labeled control DNA as described in the manual for DIG DNA Labeling Kit (Roche).

3.2.10 Extraction of Genomic DNA from Leaves

Leaf genomic DNA was isolated by the Cetyltrimethylammonium bromide (CTAB) method (Doyle *et al.*, 1990). Three pieces of leaf discs (cut by pressing tobacco leaf against the cap of an 1.5 ml microcentrifuge tube) were ground in 600 µl of 2% CTAB buffer [0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% β-mercaptoethanol (β-mer)] with a glass rod in the presence of sterile sand. The homogenate was incubated at 60°C for 40 minutes with periodic mixing. Half an ml of chloroform/ isoamyl alcohol (24:1, v/v) was added, mixed, and centrifuged at 14,000 rpm at 4°C for 15 minutes. The upper aqueous layer was transferred to a new tube and mixed with 500 µl of cold isopropanol. The mixture was kept at -20°C for 1 hour and then centrifuged at 14,000 rpm at 4°C for 30 minutes. The pellet was washed with 70% EtOH. Dried pellet was dissolved in 50 µl of TE [10 mM Tris-HCl, pH 8.0 and 1 mM EDTA] and quantified by OD_{260}.

3.2.11 PCR of Genomic DNA with P30 Specific Primers

PCR was performed to detect the presence of the P30 transgene in regenerated tobacco. A 25 μl reaction mix containing 500 ng of genomic DNA, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM P30-5' and P30-3' primers (Table 3- 1) and 1 unit of Platinum Taq DNA polymerase (Gibco) was prepared for PCR reaction using the following conditions: 94°C for 10 minutes, 30 cycles of 94°C for 20 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by 1 cycle of 72°C for 7 minutes. Five μl of PCR product was used for agarose/TAE gel electrophoresis.

3.2.12 Southern Blot Analysis of Genomic DNA

Twenty μg of genomic DNA was digested with HindIII for 16 hours, separated on 0.8% agarose/TAE gel at 45V for approximately 3 hours and transferred to a positively charged nylon membrane (Roche) using the VacuGeneXL Vacuum blotting System (Pharmacia Biotech). After UV-crosslinking, the membrane was prehybridized in Church buffer [7% SDS, 50% formamide, 5X SSC, 2% blocking reagent, 50 mM sodium phosphate, pH 7.0 and 0.1% N-lauroylsarcosine] at 42°C for 2 hours. Hybridization was carried out in Church buffer with 25 ng/ml of anti-sense DIG-labeled P30 DNA at 42°C for 16 hours. After two low stringency washes (2X SSC, 0.1% SDS) at room temperature for 15 minutes, the membrane was subjected to two high stringency washes (0.5X SSC, 0.1% SDS) at 68°C for 15 minutes.

Chemiluminescent detection was carried out with CSPD as described in the manual for DIG Luminescent Detection Kit (Roche).

3.2.13 Extraction of Total RNA from Leaves or Developing Seeds

Total RNA was extracted from leaves of transgenic plants transformed with the P30 constructs containing the constitutive promoters, pBI/35S-HP or pBI/Ubi-HP. For transgenic plants harboring seed-specific promoter constructs, pBI/Phas-HP or pBI/Phas.SP-HP, total RNA was extracted from developing seeds collected 20 days after flower (DAF). A small-scale RNA extraction protocol of Altenbach *et al.* (1989) was used.

About 0.3 g of plant tissue was ground into powder under liquid nitrogen with a mortar and pestle. After adding 0.3 ml of RNA extraction buffer [0.1 M LiCl, 0.1M Tris-HCl, pH 8.0, 0.1 M EDTA and 1% SDS] and 0.3 ml of phenol, the sample was heated at 55°C for 15 minutes. An additional 0.6 ml of chloroform/ isoamyl alcohol (24:1, v/v) was mixed with the sample and the mixture was centrifuged at 10,000 rpm for 5 minutes. The upper aqueous phase was transferred to a new tube, mixed with an equal volume of 4 M LiCl and kept at 4°C for 2 hours. The mixture was then centrifuged at 14,000 rpm at 4°C for 10 minutes. The pellet was washed with 0.2 ml of 2 M LiCl and centrifuged at 14,000 rpm at 4°C for 10 minutes. After centrifugation, the pellet was resuspended in 0.25 ml of diethylpyrocarbonate

(DEPC)-treated water and 25 μl of 3 M DEPC-treated sodium acetate (pH 5.0) before mixing with 0.5 ml of cold absolute EtOH and kept at -20°C for 2 hours. The mixture was centrifuged again at 14,000 rpm at 4°C for 10 minutes. The pellet was washed twice with 70% EtOH, allowed to air-dry and finally dissolved in 20 μl of DEPC-treated water.

3.2.14 Reverse Transcription-Polymerase Chain Reaction of Total RNA

One μg of total RNA was treated with 1 unit of RQ1 RNase-free DNase I (Promega) in a 10 μl reaction at room temperature for 15 minutes to remove DNA contamination. The RNA sample was then denatured by heating at 65°C for 5 minutes in a 20 μl mixture containing 1 μg of total RNA and 0.25 μg of Oligo $(dT)_{15}$ Primer (Promega) before cooling on ice for 5 minutes. Reverse transcription was carried out in a 40 μl reaction containing 1 μg of total RNA, 0.25 μg of Oligo dT primer, 400 units of Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (RT) (Promega), 1X M-MLV reaction buffer, 0.5 mM dNTPs, 10 mM DTT and 40 units of RNasin® Ribonuclease Inhibitor (Promega) at 42°C for 1 hour. Another set of reaction without M-MLV RT was included as an internal control. Following first strand cDNA synthesis, cDNA amplification was performed in a 25 μl reaction mixture containing 10 μl of RT product, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM 5'P30-PCR and 3'P30-PCR primers (Table 3- 1) and 1 unit of

Platinum Taq DNA polymerase (Gibco), using the following conditions: 94°C for 10 minutes, 30 cycles of 94°C for 20 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by 1 cycle of 72°C for 7 minutes. Ten μl of PCR products was used for agarose/TAE gel electrophoresis.

3.2.15 Sequencing of RT-PCR Product

PCR-aided sequencing of RT-PCR product was performed using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Kit (Applied Biosystems) to confirm the sequence fidelity of the P30 transcripts in the transgenic plants.

RT-PCR product was purified using with the Wizard PCR Preps DNA Purification System (Promega). A 10 μl sequencing reaction containing 70 ng of RT-PCR product, 0.16 pmole of P30-5'mid or P30-3'mid primer (Table 3- 1) and 4 μl of Terminator Ready Reaction Mix was performed using the following conditions: 96°C for 5 minutes, 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The sequencing product was then purified by EtOH precipitation and the pellet was resuspended in 12 μl of Template Suppression Reagent and applied to Genetic Analyzer ABI Prism 310 for analysis.

3.2.16 Northern Blot Analysis of Total RNA

Ten μg of total RNA was separated on 0.8% agarose/formaldehyde gel in 1X formaldehyde gel running buffer [40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1

mM EDTA, pH 8.0] and transferred to a positively charged nylon membrane (Roche) using the VacuGeneXL Vacuum blotting System (Pharmacia Biotech). After UV-crosslinking, the membrane was prehybridized in Church buffer [7% SDS, 50% formamide, 5X SSC, 2% blocking reagent, 50 mM sodium phosphate, pH 7.0 and 0.1% N-lauroylsarcosine] at 50°C for 2 hours. Hybridization was carried out in Church buffer with 25 ng/ml of anti-sense DIG-labeled P30 DNA at 50°C for 16 hours. After two low stringency washes (2X SSC, 0.1% SDS) at room temperature for 15 minutes, the membrane was subjected to two high stringency washes (0.5X SSC, 0.1% SDS) at 68°C for 15 minutes. Chemiluminescent detection was carried out with CSPD as described in the manual for DIG Luminescent Detection Kit (Roche).

3.2.17 Extraction of Total Protein from Leaves or Mature Seeds

Total protein was extracted from leaves of transgenic plants possessing the pBI/35S-HP or pBI/Ubi-HP construct while from mature seeds for those harboring the pBI/Phas-HP or pBI/Phas.SP-HP.

Total protein was extracted by grinding 0.3 g of plant tissue in 1 ml of protein extraction buffer [0.25 M NaCl, 0.05 M sodium phosphate, pH 8.0] with a mortar and pestle. Homogenate was then centrifuged at 14,000 rpm for 10 minutes. Clear supernatant was recovered after repeated centrifugation to remove the lipid

layer and saved as total protein extract (TPE). The bicinochoninic acid (BCA) method, using bovine serum albumin (BSA) as standard, was used to quantify TPE.

3.2.18 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

TPE (40-60 μg) was mixed with an equal volume of 2X sample loading buffer [0.0625 M Tris-HCl, pH 6.8, 2% SDS, 1% β-mer, 10% glycerol and 0.01% bromophenol blue], heated at 99°C for 5 minutes and resolved by 17.5% SDS-PAGE in electrode buffer [0.025 M Tris, 0.19 M glycine and 0.1% SDS, pH 8.3] at 30V for 1 hour and 100V for 1 hour and 30 minutes. After electrophoresis, the gel was stained in Coomassie blue solution [0.25% Coomassie brilliant blue R-250, 50% methanol and 10% glacial acetic acid] and then destained in destaining solution [10% glacial acetic acid and 50% methanol].

3.2.19 Purification of 6xHis-tagged Proteins

Purification of His-tagged proteins was based on the use of Ni-NTA Spin Kit (QIAGEN) with modifications. About 0.3 g of plant tissue was ground into homogenate in 1.5 ml of Buffer B [8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris/HCl, pH 8.0] and centrifuged at 14,000 rpm for 10 minutes. Protein extract was transferred to a pre-equilibrated Ni-NTA (nickel-nitrilotriacetic acid) spin column and centrifuged at 2,000 rpm for 2 minutes. The spin column was

washed twice with Buffer C [8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris/HCl, pH 6.3] followed by elution of the desired proteins with 100 µl of Buffer E [8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris/HCl, pH 4.5] through centrifugation at 2,000 rpm for 2 minutes.

3.2.20 Western Blot Analysis of Total Protein

Protein extract (100-200 µg) was first separated by 17.5% SDS-PAGE and blotted onto PVDF membrane (Bio-Rad) in the modified Dunn carbonate transfer buffer [10 mM $NaHCO_3$ and 3 mM Na_2CO_3] at 44V for 75 minutes using Mini Trans-Blot cell (Bio-Rad). The blot was then incubated with 6xHis Monoclonal Antibody (Clontech) at 1:5000 dilution and subjected to non-radioactive detection with chemiluminescent Starlight™ Substrate (ICN) as described in the manual of Aurora™ Western Blot Chemiluminescent Detection System (ICN).

3.2.21 *In vitro* Transcription and Translation

3.2.21.1 Construction of Transcription Vector Containing Chimeric P30 Gene

Two transcription vectors, one with a His-tag at the 5' end of the P30 coding sequence (pGEM/HP) and one without (pGEM/P), were constructed. The P30 fragment was excised from pET/E'P30F (Figure 3- 14) or pET/P30F (Figure 3- 15) and subcloned into pGEM-3Zf(+) vector (Promega) using KpnI and HindIII. The resulting constructs, with the T7 and SP6 RNA polymerase promoters flanking the target gene, were used for *in vitro* transcription.

3.2.21.2 *In vitro* Transcription

Sense P30 transcript was synthesized by *in vitro* transcription using the RiboMax^{TM} Large Scale RNA Production Systems – T7 (Promega). The transcription vectors, pGEM/HP and pGEM/P, were first linearized with HindIII. A reaction mix containing 1X Transcription buffer (Promega), 7.5 mM of each rNTP, 6 μg of linearized DNA template (or 2 μg of luciferase control DNA) and 4 μl of T7 RNA polymerase enzyme mix (Promega) was set up for each transcription reaction and incubated at 37°C for 3 hours. The quality and quantity of *in vitro* transcript was checked by electrophoresis on 1% agarose/formaldehyde gel.

3.2.21.3 *In vitro* Translation

Two *in vitro* translation systems were used, the Rabbit Reticulocyte Lysate (RL)

System and the Wheat Germ Extract (WG) System (both from Promega). Luciferase and Brome Mosaic Virus (BMV) *in vitro* transcripts were used in the positive (+ve) control reaction in the RL and WG systems, respectively. The translation reactions were as described in Tables 3- 2 and 3- 3.

The translation products were resolved in 16.5% tricine SDS-PAGE and blotted onto PVDF membrane (Bio-Rad) in the modified Dunn carbonate transfer buffer at 44V for 75 minutes using the Mini Trans-Blot cell (Bio-Rad). The blot was then subjected to non-radioactive detection with Transcend^{TM} Chemiluminescent Substrate (Promega) as described in the manual of Transcend^{TM} Non-Radioactive Translation Detection Systems (Promega).

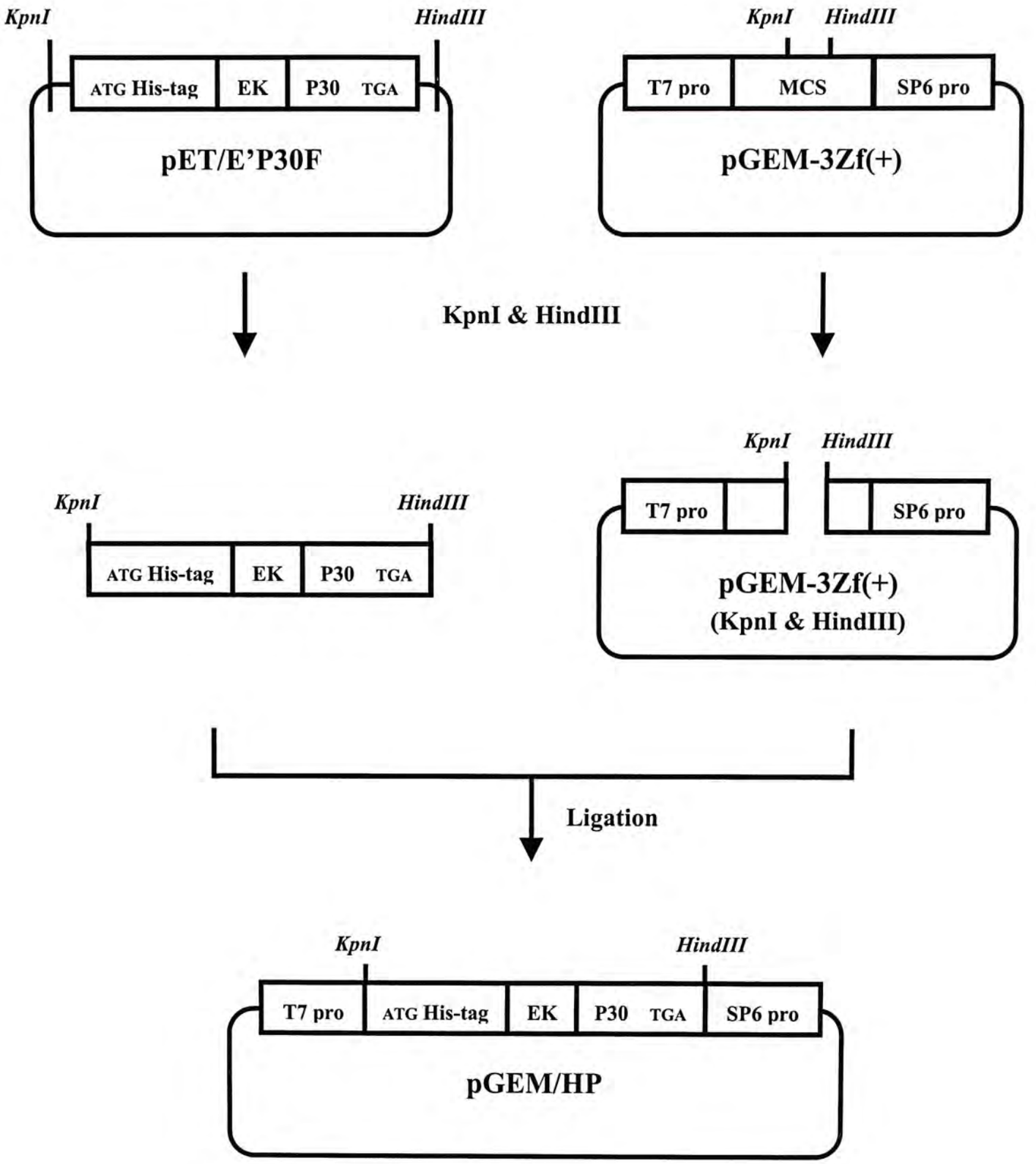


Figure 3- 14 Construction of transcription vector pGEM/HP

The P30 fragment (with a His-tag) was excised from pET/E'P30F and subcloned into pGEM-3Zf(+) with the use of KpnI and HindIII, resulting in pGEM/HP. (MCS = multiple cloning site)

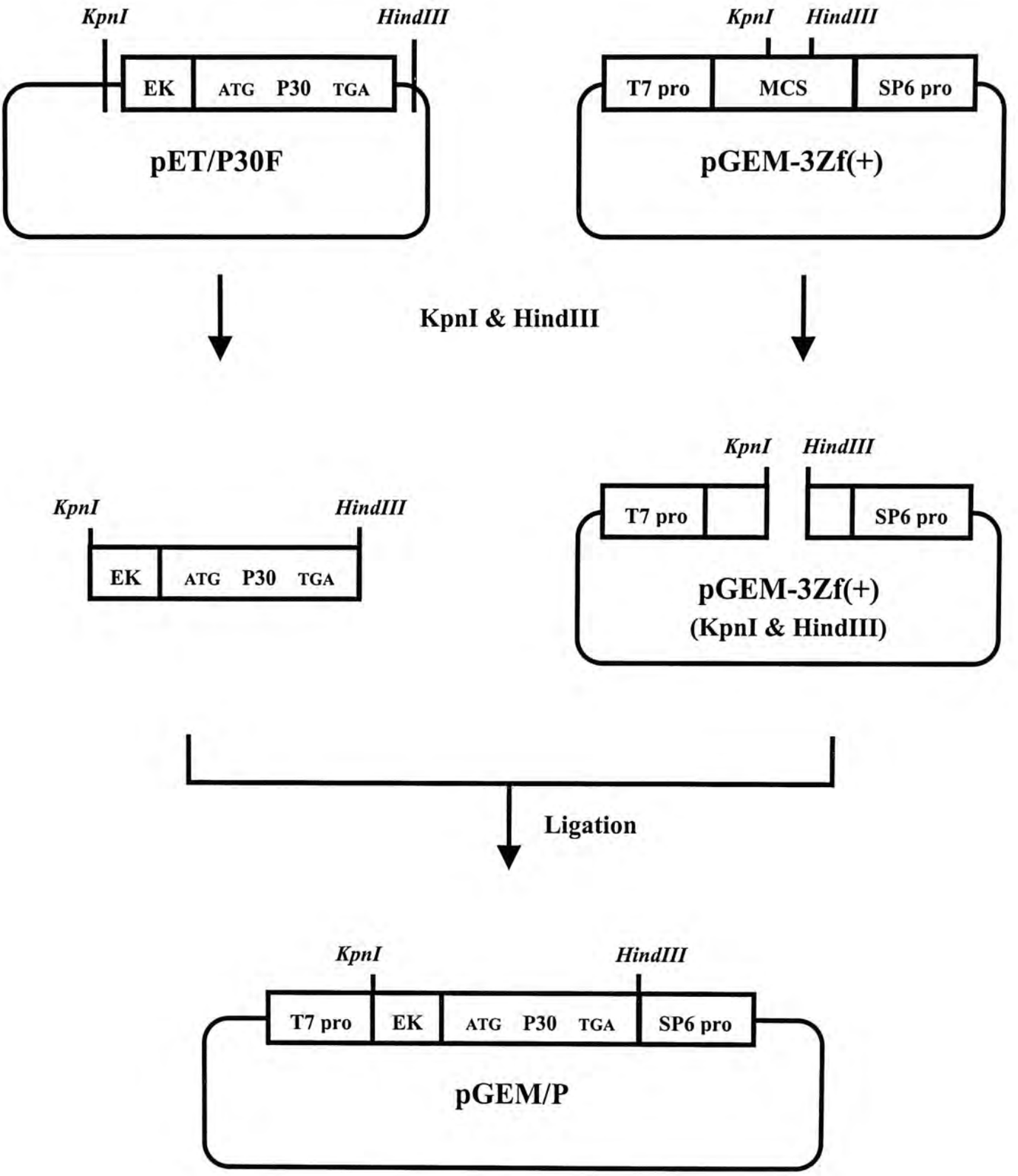


Figure 3- 15 Construction of transcription vector pGEM/P

The P30 fragment (without a His-tag) was excised from pET/DP30F and subcloned into pGEM-3Zf(+) with the use of KpnI and HindIII, resulting in pGEM/P. (MCS = multiple cloning site)

Table 3- 2 ***In vitro*** **translation reaction in the RL system**

Reagents	P30 *in vitro* transcript	+ve control (Luciferase transcript)	-ve control
RL	35 µl	35 µl	35 µl
a. a. mix – Leu	0.5 µl	0.5 µl	0.5 µl
a. a. mix – Met	0.5 µl	0.5 µl	0.5 µl
RNasin (40 u/µl)	1 µl	1 µl	1 µl
RNA	10 µg	2 µg	---
Transcend tRNA	1.5 µl	1 µl	1 µl
Total volume	50 µl	50 µl	50 µl

In the RL system, *in vitro* translation was carried out at 30°C for 90 minutes.

Table 3- 3 ***In vitro*** **translation reaction in the WG system**

Reagents	P30 *in vitro* transcript	+ve control (BWV transcript)	-ve control
WG	25 µl	25 µl	25 µl
a. a. mix – Leu	0.5 µl	0.5 µl	0.5 µl
a. a. mix – Met	0.5 µl	0.5 µl	0.5 µl
RNasin (40 u/µl)	1 µl	1 µl	1 µl
RNA	10 µg	1 µg	---
KOAc (1 M)	2 µl	3.5 µl	3.5 µl
Transcend tRNA	1.5 µl	1 µl	1 µl
Total volume	50 µl	50 µl	50 µl

In the WG system, *in vitro* translation was carried out at 25°C for 2 hours.

3.3 Results

3.3.1 Construction of Chimeric P30 Genes

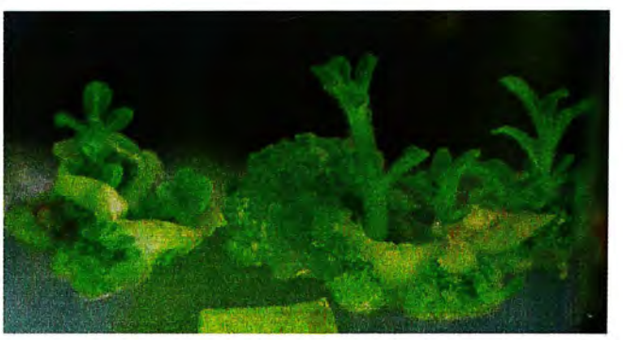
Four chimeric P30 genes, under the control of different regulatory sequences, were subcloned into the *Agrobacterium* binary vector pBI121 as described in Section 3.2.5 and transferred into *A. tumefaciens* LBA4404 through electroporation for tobacco transformation. DNA sequencing was performed to verify that the transgene constructs were correct in sequence and frame.

3.3.2 Tobacco Transformation, Selection and Regeneration

The constructs pBI/35S-HP, pBI/Ubi-HP, pBI/Phas-HP, pBI/Phas.SP-HP and pBI121 (as vector-alone control) were transformed into tobacco genome via *Agrobacterium*-mediated transformation.

Forty leaf disks wound with forceps were used for transformation. After cocultivation with *Agrobacterium* cells for 2 days, the explants were transferred to shoot regeneration medium containing kanamycin for calli and shoot formation. Regenerated shoots were transferred to root regeneration medium for rooting (Figure 3- 16). Transgenic tobacco plants were finally transferred to soil in pots and allowed to grow in greenhouse.

(A)



(B)

(C)

Figure 3- 16 Tobacco transformation, selection and regeneration

Agrobacterium tumefaciens LBA4404 harboring the target construct was used to infect tobacco leaf. Tobacco leaf explants (A) were cocultivated with *Agrobacteria* for 2 days. Under kanamycin selection, calli formed around the wounds on leaf explants and differentiated into shoots 2-4 weeks after transformation (B). Regenerated shoots were then transferred to root regeneration medium for rooting (C).

At the end, one wild type (WT) tobacco, one pBI121 transformant and three to four transformants for each chimeric P30 construct, as listed in Table 3- 4, were selected for further analysis.

Table 3- 4 Transformants selected for further analysis

Construct	Transformant	Number
pBI121	BI	1
pBI/35S-HP	S1, S2, S3	3
pBI/Ubi-HP	U1, U2, U4	3
pBI/Phas-HP	P6, P15, P17, P19	4
pBI/Phas.SP-HP	PS10, PS17, PS19, PS22	4

3.3.3 Detection of GUS Activity

Leaves from transformants were used in GUS assay. All leaves from transformants harboring the constructs showed positive results, i.e. turned deep blue while untransformed plants (WT) remained colorless after GUS staining and chlorophyll removal (Figure 3- 17), indicating that the GUS marker gene was transferred and active in transgenic plants.

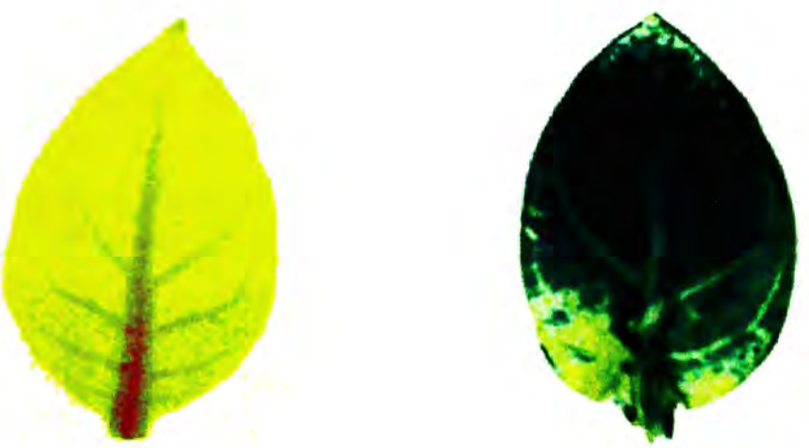


Figure 3- 17 GUS staining of tobacco leaves

Fresh tobacco leaves were placed in GUS staining solution and incubated at 37°C for color development. Chlorophyll was removed by 70% ethanol treatment. Leaf from wild type tobacco remained colorless (left) while that from transgenic one stained blue (right).

3.3.4 Detection of P30 Gene in Transgenic Plants

Besides the use of indirect means such as antibiotic selection and GUS assay, PCR and Southern blot analysis of genomic DNA were performed to confirm the integration of the P30 transgene into tobacco genome.

Genomic DNA was extracted from tobacco leaf using the CTAB method. As shown in Figure 3- 18, uncut genomic DNA appeared as one major band with molecular weight over 15 kb.

3.3.4.1 PCR of Genomic DNA

Using genomic DNA as template, PCR in the presence of the P30 specific primers (P30-5' and P30-3', Table 3- 1) was performed. A positive control using pET/P30F as template was also carried out. PCR products were resolved in 1% agarose/TAE gel. As revealed in Figure 3- 19, all the P30 transformants gave an expected PCR product of 540 bp (same as +ve control) while WT did not, indicating that the P30 transgene was present in the genome of the P30 transformants.

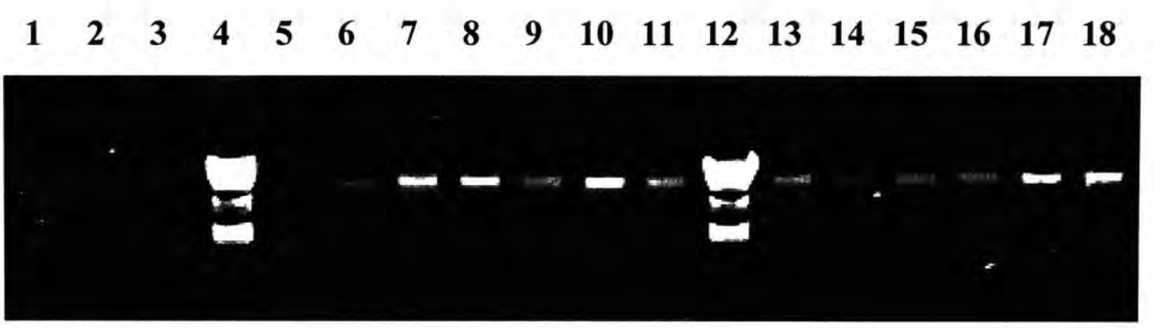


Figure 3- 18 Genomic DNA extracted from tobacco leaves using the CTAB method

Intact genomic DNA extracted from tobacco leaf was resolved in 0.7% agarose/TAE gel.

Lanes 1-3: pBI/35S-HP transformants S1, S2 and S3;
Lanes 4 & 12: High Molecular Weight DNA Markers (Gibco);
Lanes 5-7: pBI/Ubi-HP transformants U1, U2 and U4;
Lanes 8-11: pBI/Phas-HP transformants P6, P15, P17 and P19;
Lanes 13-16: pBI/Phas.SP-HP transformants PS10, PS17, PS19 and PS22;
Lane 17: Wild type (WT) tobacco;
Lane 18: pBI121 transformant BI.

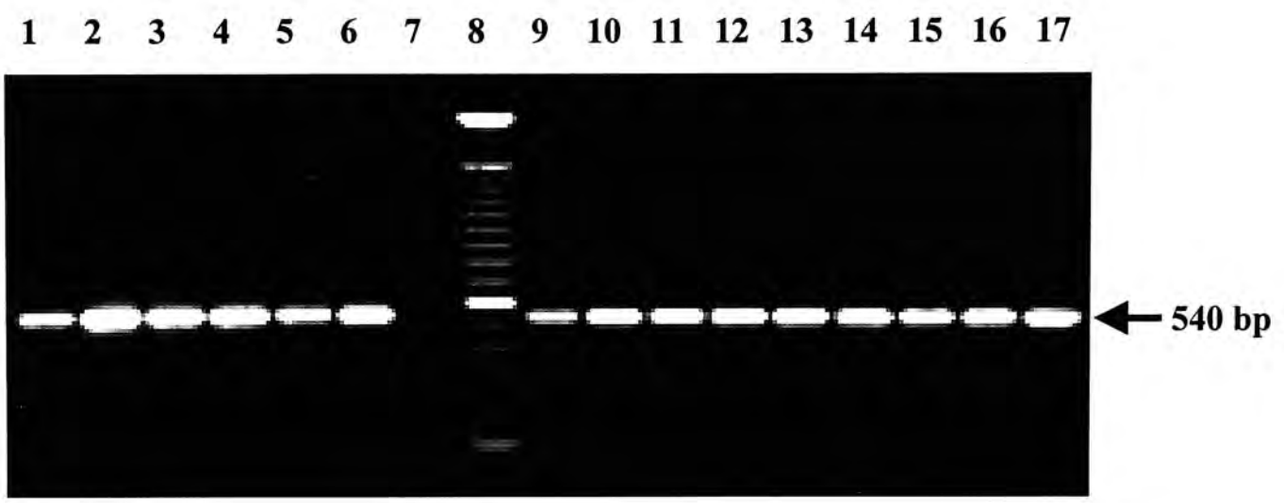


Figure 3- 19 PCR analysis of genomic DNA from wild type and transgenic tobacco plants

PCR using genomic DNA from wild type tobacco plant or P30 transformants as template was performed using the P30 specific primers P30-5' and P30-3'. PCR using plasmid pET/P30F as primer was used as positive (+ve) control. The expected size of PCR product was 540 bp.

Lanes 1-3: pBI/35S-HP transformants S1, S2 and S3;
Lanes 4-6: pBI/Ubi-HP transformants U1, U2 and U4;
Lane 7: Wild type (WT) tobacco;
Lane 8: 100 bp DNA ladder (Gibco);
Lane 9: +ve control (pET/P30F);
Lanes 10-13: pBI/Phas-HP transformants P6, P15, P17 and P19;
Lanes 14-17: pBI/Phas.SP-HP transformants PS10, PS17, PS19 and PS22.

3.3.4.2 Southern Blot Analysis

The integration of chimeric P30 gene into tobacco genome was further confirmed by Southern blot analysis. Genomic DNA was cut with HindIII, separated on 0.8% agarose/TAE gel and blotted on positively charged nylon membrane. After hybridization with anti-sense DIG-labeled P30 DNA probe, chemiluminescent detection was carried out with CSPD.

As illustrated in Figure 3- 20, all the pBI/Phas-HP and pBI/Phas.SP transformants revealed a signal of about 3.2 kb, as in the positive control (plasmid pBI/Phas-HP cut with HindIII). For transformants pBI/35S-HP and pBI/Ubi-HP, positive signals with expected sizes (about 3.1 kb and 3.5 kb, respectively) were detected (Figure 3- 21). No signal was detected in the wild type plant or pBI121 transformant, showing that the signals were not due to non-specific binding of the probe. These results further confirmed the integration and presence of the transgenes in the genome of the P30 transformants.

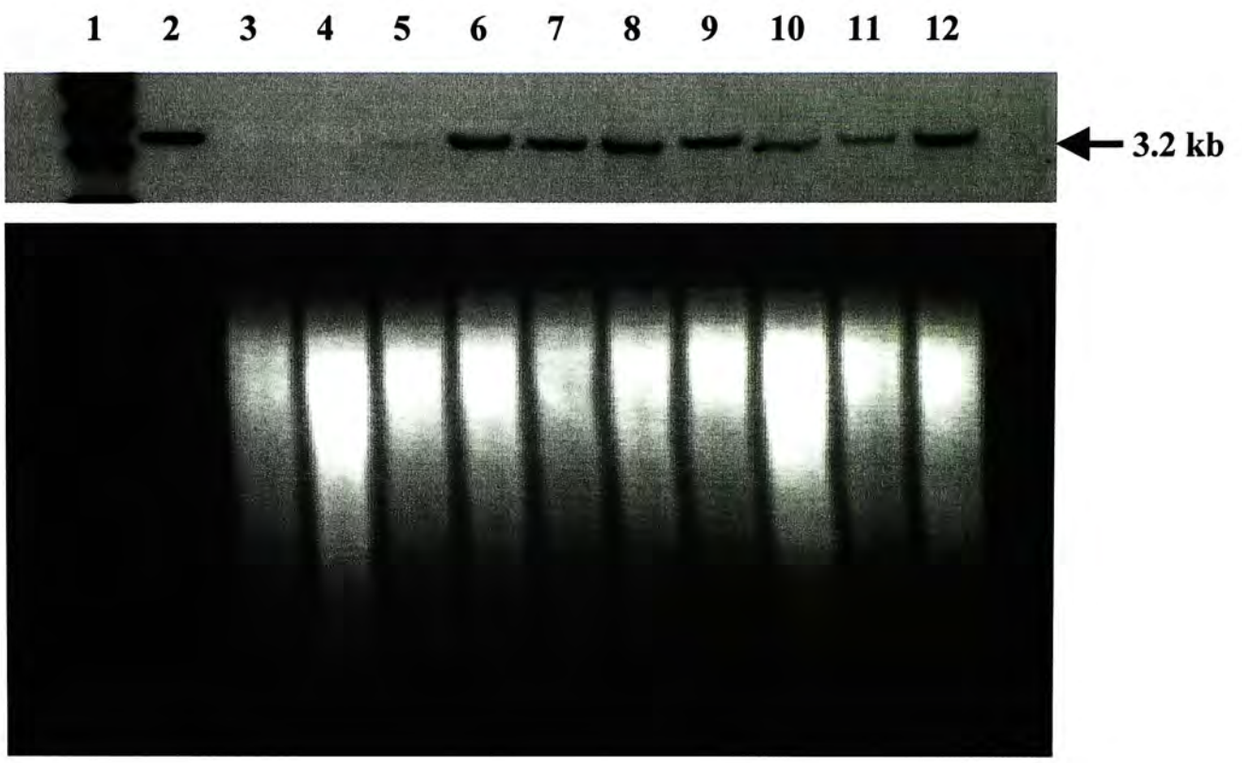


Figure 3- 20 Southern blot analysis of genomic DNA from transformants P and PS

Genomic DNA extracted from tobacco leaves was digested with HindIII, separated on 0.8% agarose/TAE gel (lower panel), blotted on positively charged nylon membrane and hybridized with DIG-labeled P30 probe (upper panel).

Lane 1:	DNA molecular weight VII, DIG-labeled (Roche);
Lane 2:	+ve control (150 pg of pBI/Phas-HP digested with HindIII);
Lane 3:	Wild type (WT) tobacco;
Lane 4:	pBI121 transformant BI;
Lanes 5-8:	pBI/Phas-HP transformants P6, P15, P17 and P19;
Lanes 9-12:	pBI/Phas.SP-HP transformants PS10, PS17, PS19 and PS22.

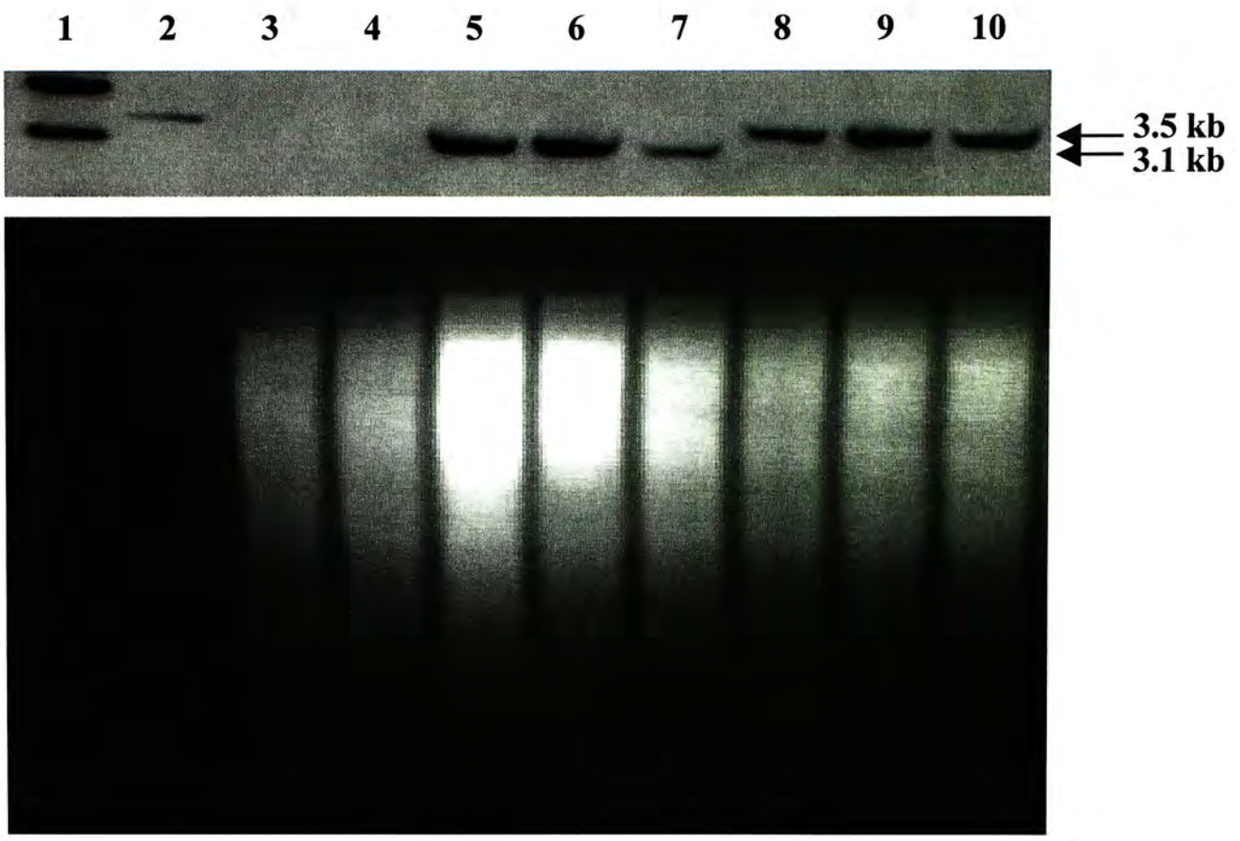


Figure 3- 21 Southern blot analysis of genomic DNA from transformants S and U

Genomic DNA extracted from tobacco leaves was digested with HindIII, separated on 0.8% agarose/TAE gel (lower panel), blotted on positively charged nylon membrane and hybridized with DIG-labeled P30 probe (upper panel).

Lane 1: DNA molecular weight VII, DIG-labeled (Roche);
Lane 2: +ve control
(150 pg of pBI/35S-HP digested with HindIII);
Lane 3: Wild type (WT) tobacco;
Lane 4: pBI121 transformant BI;
Lanes 5-7: pBI/35S-HP transformants S1, S2 and S3;
Lanes 8-10: pBI/Ubi-HP transformants U1, U2 and U4.

3.3.5 Detection of P30 Transcript in Transgenic Plants

Total RNA was extracted from the leaves of transformants harboring the constitutive promoter constructs (pBI/35S-HP or pBI/Ubi-HP). Since the phaseolin promoter is seed-specific and temporarily regulated, total RNA for transformants harboring the pBI/Phas-HP or pBI/Phas.SP-HP construct was extracted from developing seeds collected 20 DAF instead. RT-PCR, sequencing of the RT-PCR products and northern blot analysis were performed to detect and confirm the presence and sequence fidelity of the P30 transcripts in these transformants.

3.3.5.1 RT-PCR

DNase I treatment of total RNA was first performed to eliminate any DNA contamination which may lead to false positive results in the second strand cDNA PCR. In the RT reaction, Oligo $(dT)_{15}$ Primer (Promega) was used and a set of reaction without M-MLV RT (lanes with -) was included as an internal control. Following cDNA synthesis, PCR with the P30 specific primers, 5'P30-PCR and 3'P30-PCR, was carried out. Ten μl of PCR product was used for agarose/TAE gel electrophoresis.

As revealed in Figures 3- 22 and 3- 23, all the pBI/Phas-HP and pBI/35S-HP transformants showed positive results in the RT-PCR with a band of 740 bp, as in +ve control (using pET/E'P30F as the template). However, like WT, no P30

transcript was detected in the pBI/Ubi-HP transformants.

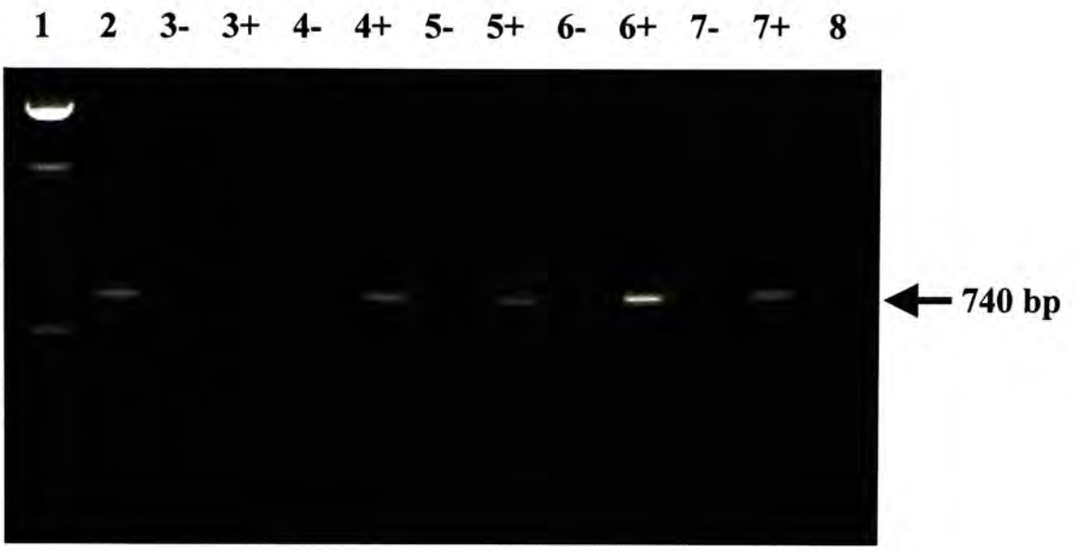


Figure 3- 22 RT-PCR of total RNA from transformants P

Total RNA was first treated with DNase I to remove DNA contaminant. RT reaction was first performed with oligo dT primer while the second strand cDNA PCR used the P30 specific primers 5'P30-PCR and 3'P30-PCR.

Lane 1: 100 bp DNA ladder (Gibco);
Lane 2: +ve control
(PCR of plasmid pET/E'P30F);
Lanes 3: Wild type (WT) tobacco;
Lanes 4-7: pBI/Phas-HP transformant P6, P15, P17 and P19;
Lane 8: -ve control
(PCR without template);
Lanes with +: RT reaction with M-MLV RT;
Lanes with -: RT reaction without M-MLV RT (internal control).

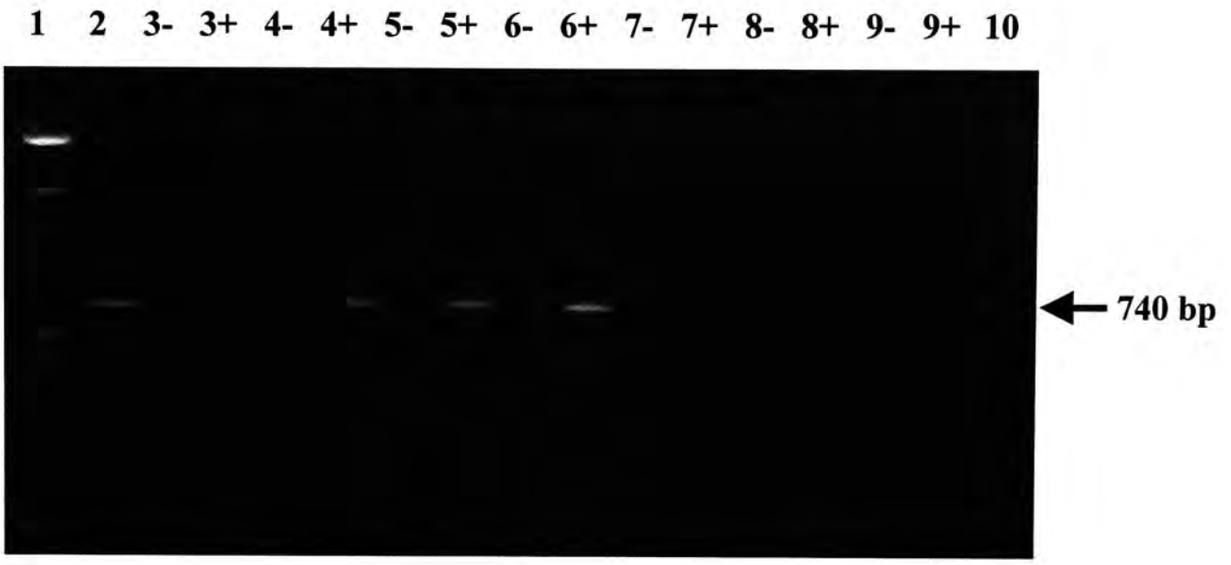


Figure 3- 23 RT-PCR of total RNA from transformants S and U

Total RNA was first treated with DNase I to remove any DNA contaminant. RT reaction was first performed with oligo dT primer while the second strand cDNA PCR used the P30 specific primers 5'P30-PCR and 3'P30-PCR.

Lane 1: 100 bp DNA ladder (Gibco);
Lane 2: +ve control
(PCR of plasmid pET/E'P30F);
Lanes 3: Wild type(WT) tobacco;
Lanes 4-6: pBI/35S-HP transformant S1, S2 and S3;
Lanes 7-9: pBI/Ubi-HP transformant U1, U2 and U4;
Lane 10: -ve control
(PCR without template);
Lanes with +: RT reaction with M-MLV RT;
Lanes with -: RT reaction without M-MLV RT (internal control).

3.3.5.2 Sequencing of RT-PCR Product

The fidelity of the P30 transcript was further confirmed by DNA sequencing. Purified RT-PCR products from P17 and S2 were used as template for cycle sequencing using the P30 internal primer P30-5'mid or P30-3'mid. Sequencing products were applied to automatic DNA sequencer for analysis. Total agreement between the sequencing data and the original P30 sequence further confirmed the presence of the P30 transcription in transformants P and S.

3.3.5.3 Northern Blot Analysis

Total RNA extracted either from tobacco developing seeds or leaves was resolved in 0.8% agarose/formaldehyde gel, blotted onto positively charged nylon membrane and hybridized with anti-sense DIG-labeled P30 probe. The P30 transcript HP (0.82 kb) synthesized via *in vitro* transcription (Section 3.2.21.2) was served as a positive control.

As revealed in Figures 3- 24 and 3- 25, all the pBI/Phas.SP-HP (PS10, PS17, PS19 and PS22), pBI/35S-HP (S1, S2 and S3) and two of the pBI/Phas-HP (P15 and P19) transformants showed positive signals in the northern blot analysis. No signal was detected in the pBI/Ubi-HP transformants, in agreement with the RT-PCR results shown in Section 3.3.5.1. Transformants PS19, PS22, S1 and S3 appeared to have relatively higher levels of production of the P30 transcripts in comparison to

other P30 transformants.

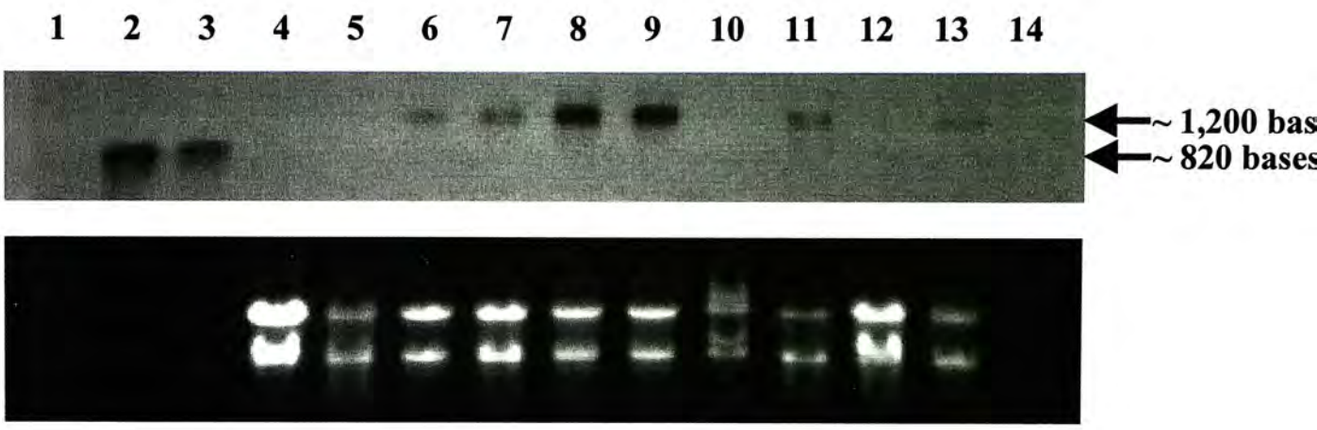


Figure 3- 24 Northern blot analysis of total RNA from transformants P and PS

Total RNA extracted from tobacco developing seeds was separated on 0.8% agarose/formaldehyde gel (lower panel), blotted onto positively charged nylon membrane and hybridized with the DIG-labeled P30 probe (upper panel).

Lanes 1 & 14: RNA Markers, 0.28-6.58 kb (Promega);

Lanes 2 & 3: +ve control
(150 & 100 ng of *in vitro* P30 transcript HP with a size about 820 bases);

Lane 4: Wild type (WT) tobacco;

Lane 5: pBI121 transformant BI;

Lanes 6-9: pBI/Phas.SP-HP transformants PS10, PS17, PS19 and PS22;

Lanes 10-13: pBI/Phas-HP transformants P6, P15, P17 and P19.

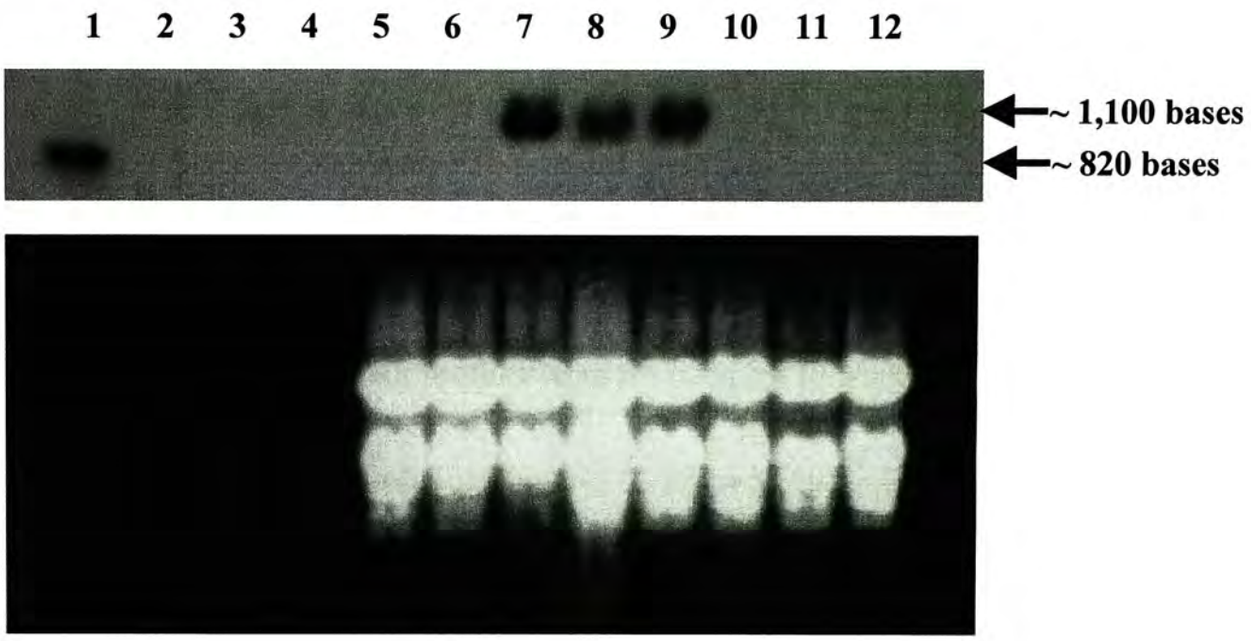


Figure 3- 25 Northern blot analysis of total RNA from transformants S and U

Total RNA extracted from tobacco leaves was separated on 0.8% agarose/formaldehyde gel (lower panel), blotted onto positively charged nylon membrane and hybridized with the DIG-labeled P30 probe (upper panel).

Lane 1: +ve control
(100 ng of *in vitro* P30 transcript HP with a size about 820 bases);
Lanes 2 & 4: Blank;
Lane 3: RNA Markers, 0.28-6.58 kb (Promega);
Lane 5: Wild type (WT) tobacco;
Lane 6: pBI121 transformant BI;
Lanes 7-9: pBI/35S-HP transformants S1, S2 and S3;
Lanes 10-12: pBI/Ubi-HP transformants U1, U2 and U4.

3.3.6 Detection of P30 Protein in Transgenic Plants

Total protein was extracted with phosphate extraction buffer from mature seeds of pBI/Phas-HP and pBI/Phas.SP transformants or from leaves of pBI/35S-HP transformants. Since recombinant P30 proteins were His-tagged, the Ni-NTA spin kit was also used to purify and concentrate any possible His-tagged proteins in the transgenic samples. The protein profiles were revealed by 17.5% SDS-PAGE after staining with Coomassie blue solution (Figures 3- 26 to 3- 29). Recombinant P30 proteins should have molecular weights around 26 kDa to 29 kDa. However, no obvious difference could be observed between the protein profiles of wild type, pBI121 transformants and the P30 transformants. As the amounts of recombinant P30 proteins might not be sufficiently abundant to detect by Coomassie blue staining, more sensitive and precise western blot analysis was performed.

3.3.6.1 Western Blot Analysis of Total Protein and Ni-NTA Purified Proteins

Protein extract or purified His-tagged protein (100-200 μg) was separated by 17.5% SDS-PAGE, blotted onto PVDF membrane and incubated with 6xHis Monoclonal Antibody (Clontech) at 1:5000 dilution. A 6xHis ladder (QIAGEN) was served as both molecular size marker and positive control. After chemiluminescent detection, the positive control gave a strong signal but no signal was found in the P30 transformants (Figures 3- 30 to 3- 33).

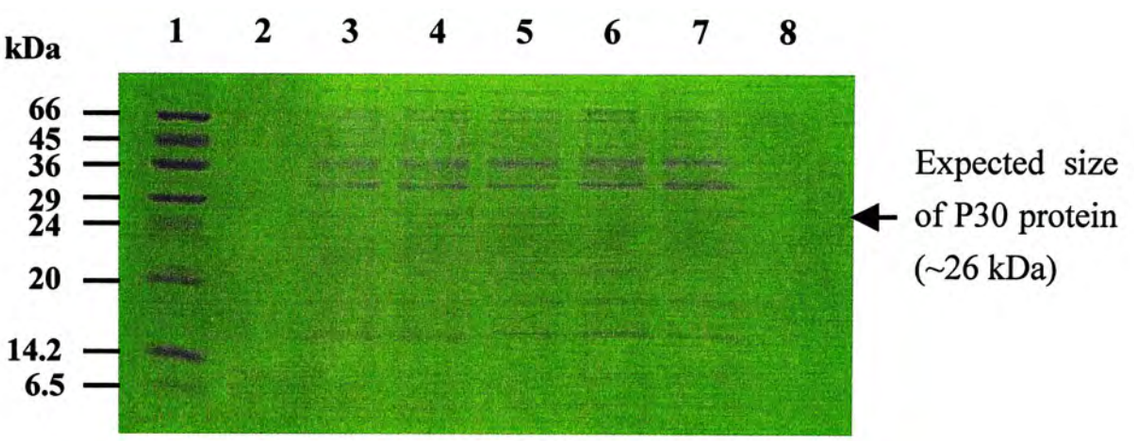


Figure 3- 26 SDS-PAGE of total protein from tobacco leaves

Total protein (60 μg) was extracted from tobacco leaves, resolved in 17.5% SDS-PAGE and stained with Coomassie blue.

Lane 1: SIGMAMARKER Low Molecular Weight Range (Sigma);

Lanes 2 & 8: Blank;

Lane 3: Wild type (WT) tobacco;

Lane 4: pBI121 transformant BI;

Lanes 5-7: pBI/35S-HP transformants S1, S2 and S3.

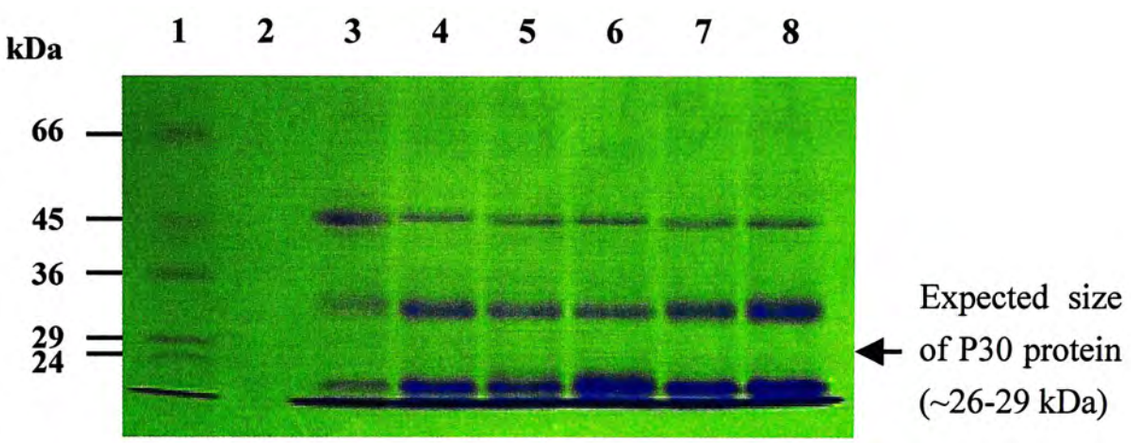


Figure 3- 27 SDS-PAGE of total protein from tobacco mature seeds

Total protein (40 μg) was extracted from tobacco mature seeds, resolved in 17.5% SDS-PAGE and stained with Coomassie blue.

Lane 1: SIGMAMARKER Low Molecular Weight Range (Sigma);
Lane 2: Blank;
Lane 3: Wild type (WT) tobacco;
Lane 4: pBI121 transformant BI;
Lanes 5 & 6: pBI/Phas-HP transformants P15 and P19;
Lanes 7 & 8: pBI/Phas.SP-HP transformants PS19 and PS22.

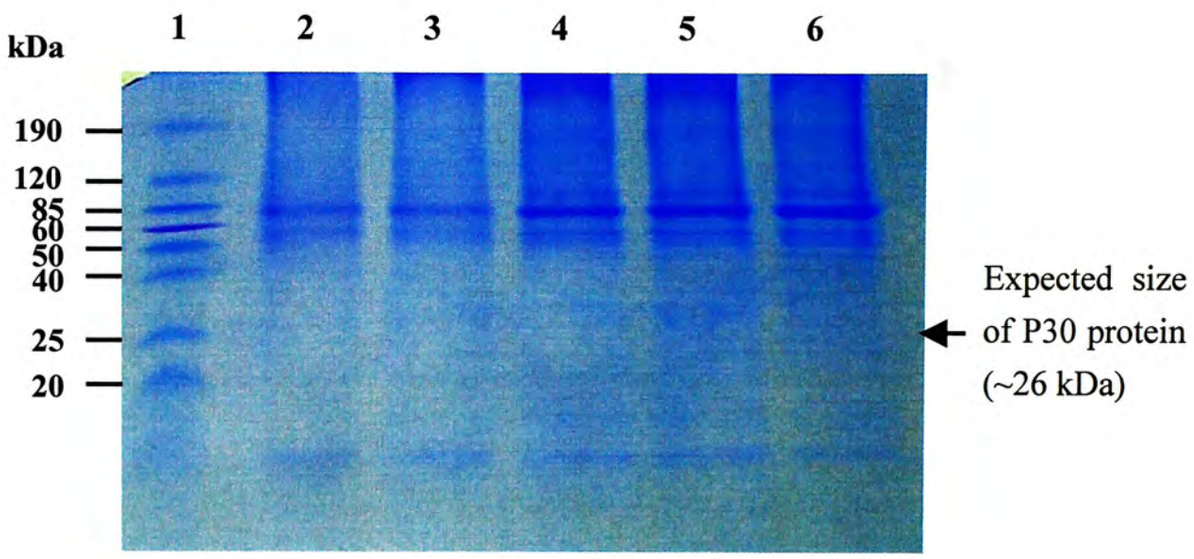


Figure 3- 28 SDS-PAGE of Ni-NTA purified proteins from tobacco leaves

Ni-NTA purified proteins were obtained from tobacco leaf extract after interaction with Ni-NTA spin column, resolved in 17.5% SDS-PAGE and stained with Coomassie blue.

Lane 1: BenchMark Prestained Protein Ladder (Gibco);

Lane 2: Wild type (WT) tobacco;

Lane 3: pBI121 transformant BI;

Lanes 4-6: pBI/35S-HP transformants S1, S2 and S3.

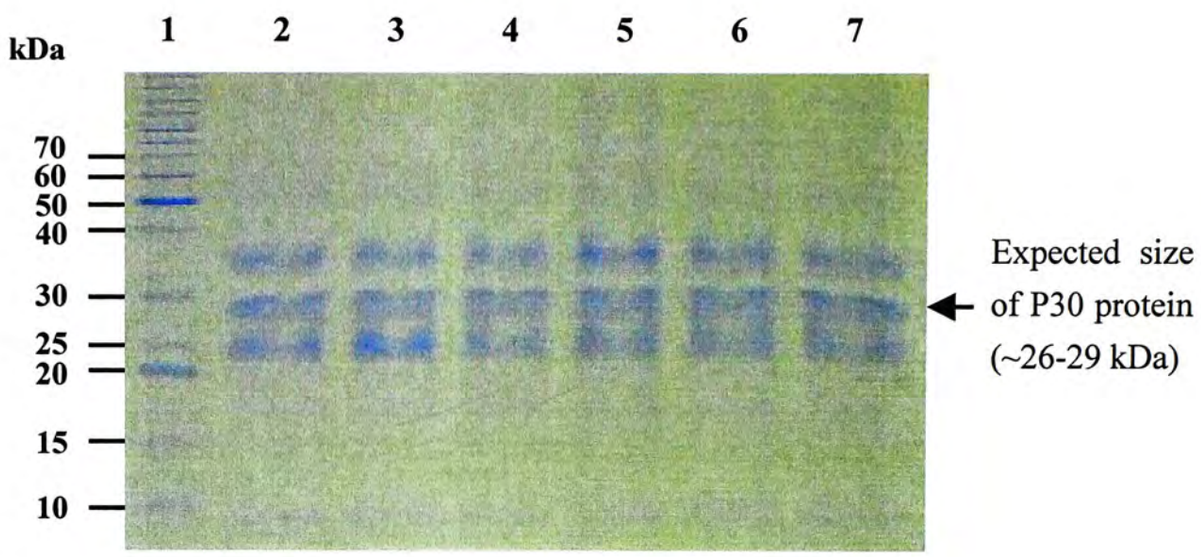


Figure 3- 29 SDS-PAGE of Ni-NTA purified proteins from tobacco seeds

Ni-NTA purified proteins were obtained from tobacco mature seeds after interaction with Ni-NTA spin column, resolved in 17.5% SDS-PAGE and stained with Coomassie blue.

Lane 1: BenchMark Protein Ladder (Gibco);
Lane 2: Wild type (WT) tobacco;
Lane 3: pBI121 transformant BI;
Lanes 4 & 5: pBI/Phas-HP transformants P15 and P19;
Lanes 6 & 7: pBI/Phas.SP-HP transformants PS19 and PS22.

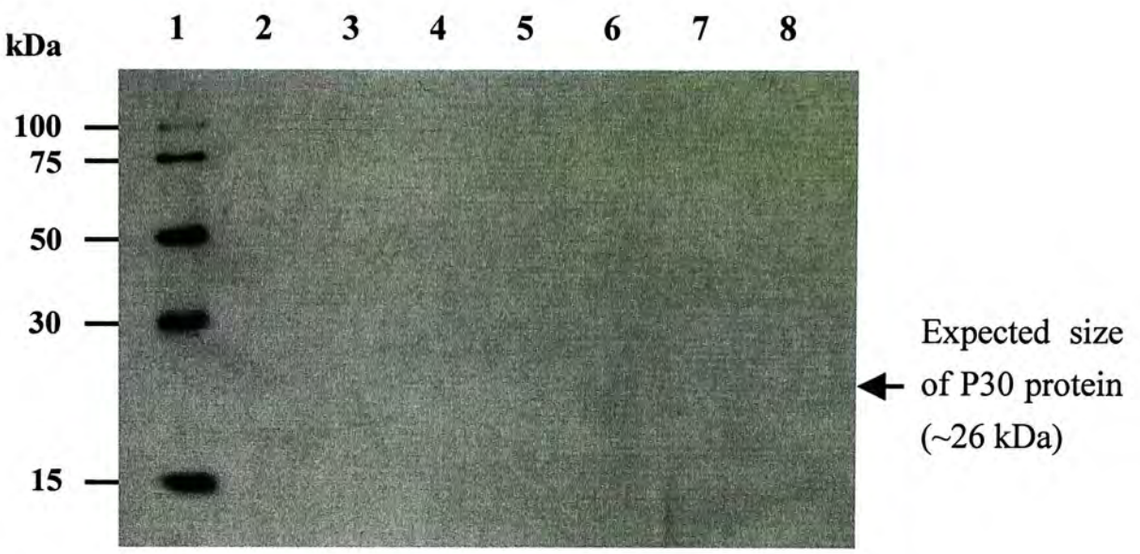


Figure 3- 30 Western blot analysis of total protein from tobacco leaves

Total protein (150 μg) was extracted from tobacco leaves, resolved in 17.5% SDS-PAGE and blotted on PVDF membrane. Western blot analysis using 6xHis Monoclonal Antibody (Clontech) was carried out.

Lane 1: 6xHis ladder (QIAGEN);
Lanes 2 & 8: Blank;
Lane 3: Wild type (WT) tobacco;
Lane 4: pBI121 transformant BI;
Lanes 5-7: pBI/35S-HP transformants S1, S2 and S3.

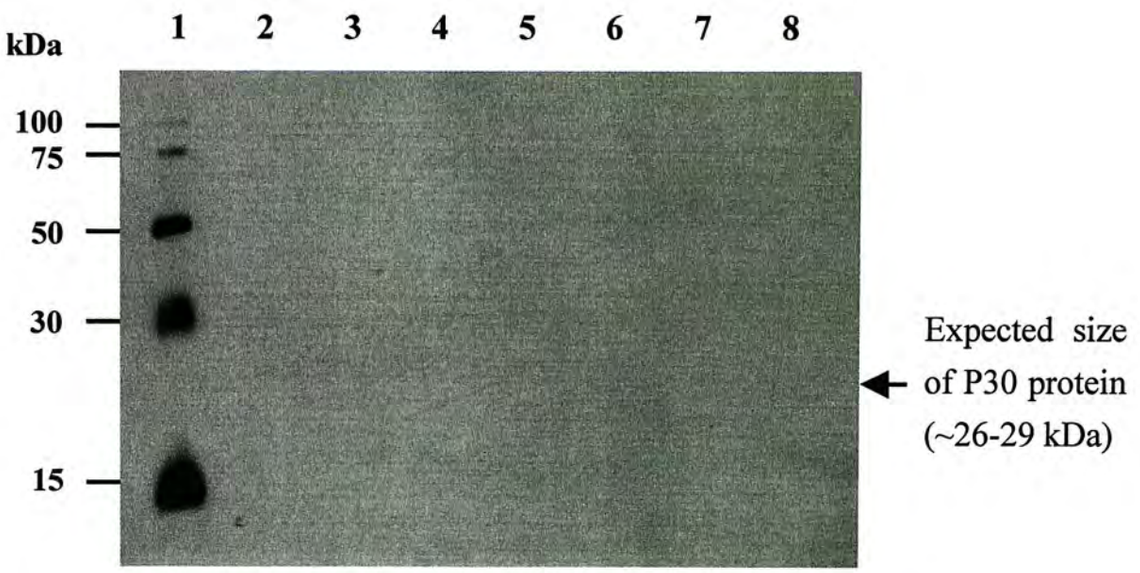


Figure 3- 31 Western blot analysis of total protein from tobacco seeds

Total protein (200 μg) was extracted from tobacco mature seeds, resolved in 17.5% SDS-PAGE and blotted on PVDF membrane. Western blot analysis using 6xHis Monoclonal Antibody (Clontech) was carried out.

Lane 1: 6xHis ladder (QIAGEN);
Lane 2: Blank;
Lane 3: Wild type (WT) tobacco;
Lane 4: pBI121 transformant BI;
Lanes 5 & 6: pBI/Phas-HP transformants P15 and P19;
Lanes 7 & 8: pBI/Phas.SP-HP transformants PS19 and PS22.

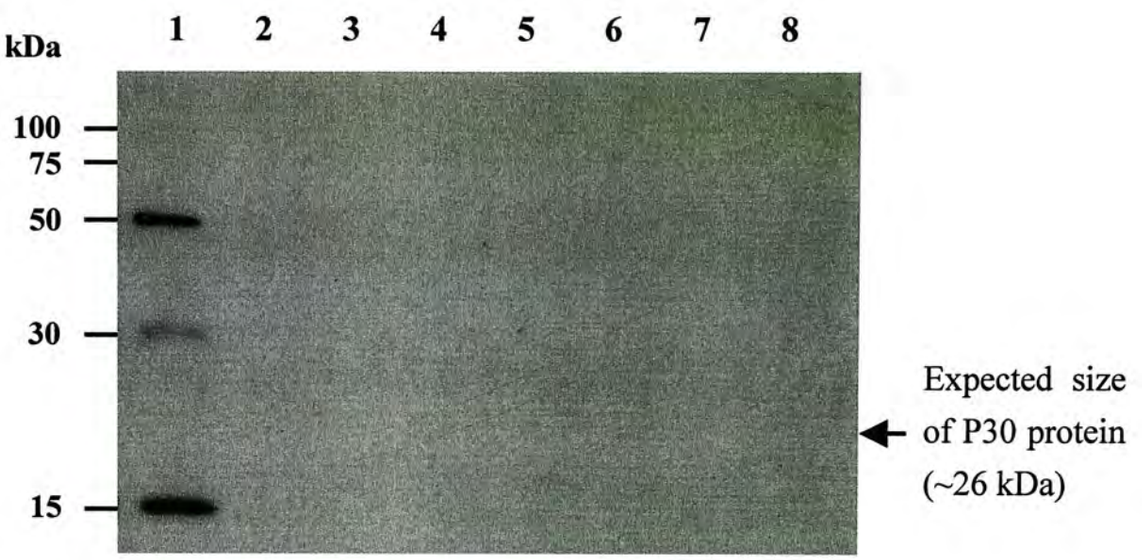


Figure 3- 32 Western blot analysis of Ni-NTA purified proteins from tobacco leaves

Ni-NTA purified proteins (200 μg) were obtained from tobacco leaves after reaction with Ni-NTA spin column, resolved in 17.5% SDS-PAGE and blotted on PVDF membrane. Western blot analysis using 6xHis Monoclonal Antibody (Clontech) was carried out.

Lane 1: 6xHis ladder (QIAGEN);
Lanes 2 & 8: Blank;
Lane 3: Wild type (WT) tobacco;
Lane 4: pBI121 transformant BI;
Lanes 5-7: pBI/35S-HP transformants S1, S2 and S3.

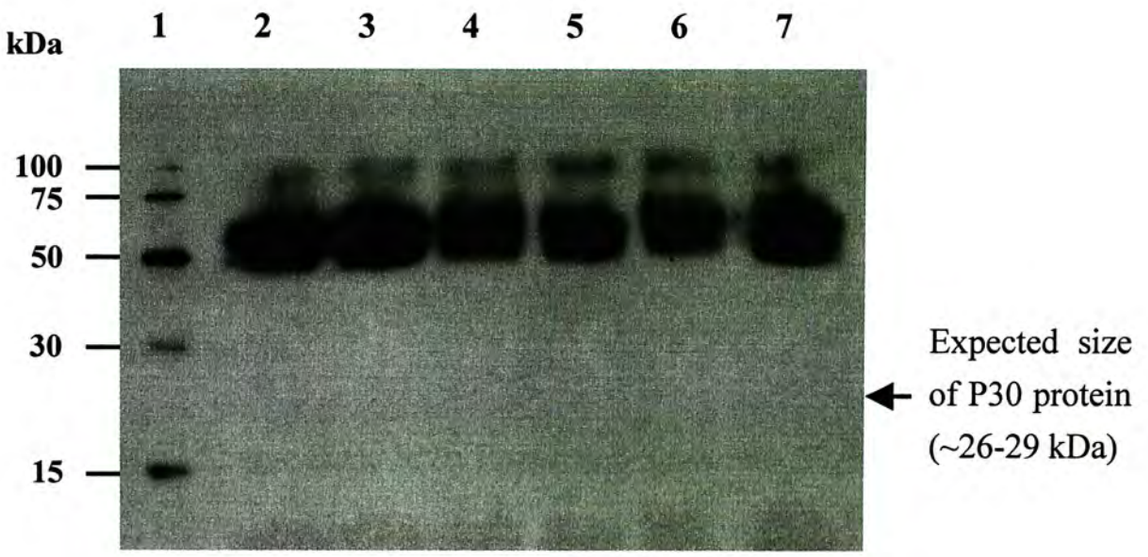


Figure 3- 33 Western blot analysis of Ni-NTA purified proteins from tobacco seeds

Ni-NTA purified proteins (200 μg) were obtained from tobacco mature seeds after reaction with Ni-NTA spin column, resolved in 17.5% SDS-PAGE and blotted on PVDF membrane. Western blot analysis using 6xHis Monoclonal Antibody (Clontech) was carried out.

Lane 1: 6xHis ladder (QIAGEN);
Lane 2: Wild type (WT) tobacco;
Lane 3: pBI121 transformant BI;
Lanes 4 & 5: pBI/Phas-HP transformants P15 and P19;
Lanes 6 & 7: pBI/Phas.SP-HP transformants PS19 and PS22.

3.3.7 *In vitro* Transcription and Translation

In vitro transcription and translation were performed to further investigate the translatability of chimeric P30 transcripts.

Two transcription vectors, one with a His-tag at the 5' end of the P30 coding sequence (pGEM/HP) and one without (pGEM/P), were constructed as described in Section 3.2.21.1.

3.3.7.1 *In vitro* Transcription

Using the RiboMax™ Large Scale RNA Production Systems – T7, sense P30 *in vitro* transcripts HP and P with sizes of about 0.82 kb and 0.8 kb were generated from HindIII-linearized pGEM/HP and pGEM/P, respectively. Luciferase control DNA was used as +ve control and its *in vitro* transcript was 1.8 kb in length (Figure 3- 34).

3.3.7.2 *In vitro* Translation

In vitro translation was conducted in 2 systems: an animal cell-free translation system, rabbit reticulocyte lysate (RL) and a plant system, wheat germ extract (WG). During translation, biotinylated lysine residues (as a precharged ε-labeled biotinylated lysine-tRNA complex named Transcend™ tRNA) were incorporated into nascent proteins. The translation products were resolved in 16.5% tricine SDS-PAGE and blotted on PVDF membrane. Non-radioactive detection with

Streptavidin-Horseradish Peroxidase was carried out to visualize the biotinylated proteins.

The expected sizes of *in vitro* translation products of the P30 *in vitro* transcripts HP and P would be 25.7 kDa and 24.1 kDa, respectively. As shown in Figure 3- 35, in the RL system, luciferase *in vitro* transcript (+ve control) gave an expected band of 61 kDa. Using the *in vitro* produced P30 transcripts, *in vitro* translation products of expected sizes could also be detected. However, in the WG system, only the expected translation products of BMV with a size of 35 kDa was detected. No signal was found for the P30 *in vitro* transcripts (Figure 3- 36). The results suggest that the translation of the P30 transcript may not be efficient in the plant system.

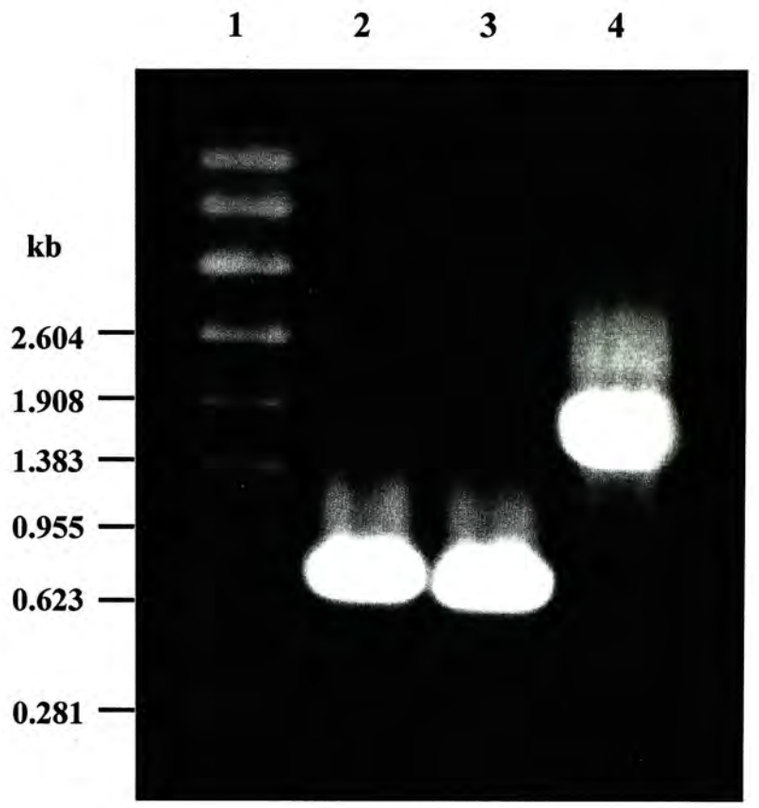


Figure 3- 34 Transcripts synthesized in *in vitro* transcription

In vitro transcripts were resloved in 1% agarose/formaldehyde gel.

Lane 1: RNA Markers, 0.28-6.58 kb (Promega);

Lane 2: *In vitro* transcript HP (from pGEM/HP);

Lane 3: *In vitro* transcript P (from pGEM/P);

Lane 4: Luciferase *in vitro* transcript (+ve control).

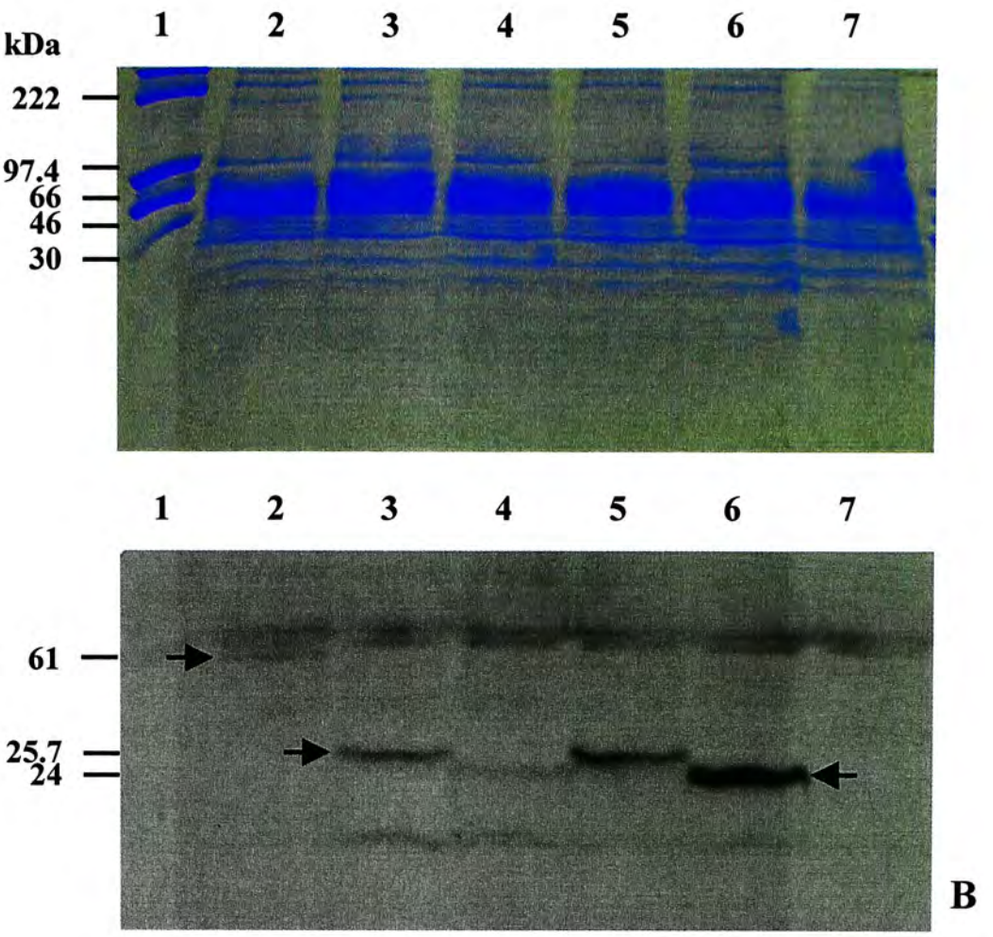


Figure 3- 35 Tricine SDS-PAGE (A) and non-radioactive detection (B) of *in vitro* translation products from the RL system

In vitro translation products from the RL system were resolved in 16.5% tricine SDS-PAGE and stained with Coomassie blue (Panel A) or blotted onto PVDF membrane for non-radioactive detection with Streptavidin-Horseradish Peroxidase to visualize the biotinylated proteins (Panel B).

Lane 1: Rainbow™ coloured protein molecular weight markers, High molecular weight range (Amersham Pharmacia);
Lane 2: +ve control (luciferase *in vitro* translation product);
Lanes 3 & 5: *in vitro* translation product HP (from pGEM/HP);
Lanes 4 & 6: *in vitro* translation product P (from pGEM/P);
Lane 7: -ve control (no transcript added in translation).

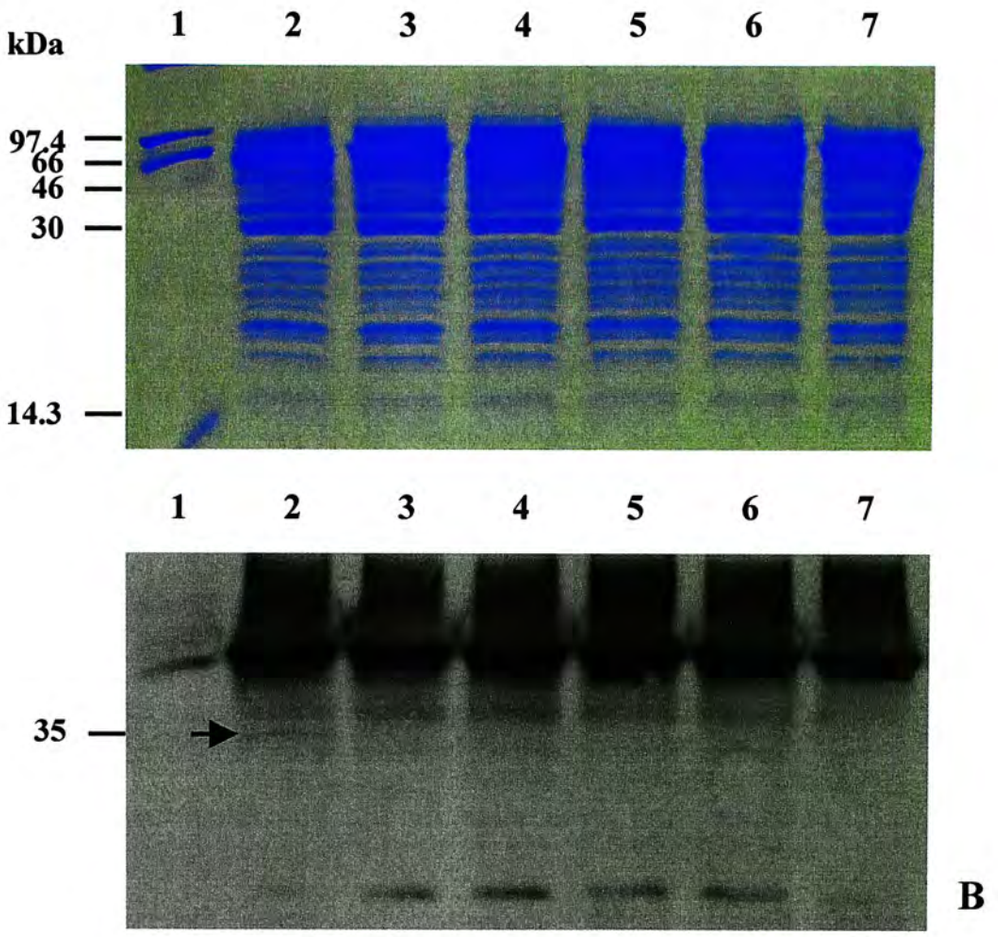


Figure 3- 36 Tricine SDS-PAGE (A) and non-radioactive detection (B) of *in vitro* translation products from the WG system

In vitro translation products from the WG system were resolved in 16.5% tricine SDS-PAGE and stained with Coomassie blue (Panel A) or blotted onto PVDF membrane for non-radioactive detection with Streptavidin-Horseradish Peroxidase to visualize the biotinylated proteins (Panel B).

Lane 1: Rainbow™ coloured protein molecular weight markers, High molecular weight range (Amersham Pharmacia);
Lane 2: +ve control (BMV *in vitro* translation product);
Lanes 3 & 5: *in vitro* translation product HP (from pGEM/HP);
Lanes 4 & 6: *in vitro* translation product P (from pGEM/P);
Lane 7: -ve control (no transcript added in translation).

Chapter 4 Discussion

4.1 General Conclusion

P30 is a major surface antigen from a widespread intracellular parasite *T. gondii* that is responsible for toxoplasmosis. Because of its importance in studying the immune and pathogenic mechanisms of the parasite and in possible development of subunit vaccine, production of large amounts of high quality P30 by recombinant DNA technology becomes valuable. Successful expression of the P30 protein in bacteria, yeast, insect cells and animal cells have been reported (Section 2.3). However, so far, there has been no publication on P30 expression in plants.

With the advances in plant biotechnology using plants as bioreactors to produce recombinant proteins such as antibodies and vaccines in bulk amounts at relatively low costs generates much commercial interest. Plant protein expression system shows several important advantages over other heterologous expression systems since plants can be grown easily and inexpensively in large scale. Moreover, scale-up, harvesting, transporting, storage and processing of plants can be simple and efficient with the available agronomic infrastructures (Fischer *et al.*, 1999). The presence of nearly all post-translational modifications essential for optimal biological activity of proteins is also an advantage of plants in expressing eukaryotic genes.

Furthermore, safe recombinant proteins can be obtained, as contamination of expressed proteins with human or animal pathogens or other infectious diseases will not occur in plants (Fischer and Emans, 2000). Despite all the above advantages, introducing a foreign gene into a plant host does not always result in high-level expression or even expression of the introduced gene at all. Effective use of plants as bioreactors thus depends on the ability to obtain high protein accumulation levels that are stable during the life cycle of a transgenic plant and in subsequent generations.

This work reports the first use of tobacco plants to express P30 of *T. gondii*. In the study, chimeric P30 genes under the control of the CaMV 35S, Ubi or phaseolin (with or without SP) promoter were constructed and introduced into tobacco via *Agrobacterium*-mediated transformation. Regenerated plants were first selected based on their kanamycin resistance (NPTII gene expression) and then screened with GUS assay (GUS gene expression). In genomic DNA PCR (Figure 3- 19) and Southern blot analysis (Figures 3- 20 and 3- 21), all the P30 transformants showed positive results with correct fragment size confirming the integration of the P30 transgene into tobacco genome without any sequence rearrangement. The presences of the P30 transcripts in some of the P30 transformants including the pBI/Phas.SP-HP transformants PS10, PS17, PS19 and PS22; pBI/35S-HP

transformants S1, S2 and S3; and pBI/Phas-HP transformants P15 and P19, were revealed by RT-PCR (Figures 3- 22 and 3- 23) and northern blot analysis (Figures 3- 24 and 3- 25). The fidelity of the P30 transcripts was further confirmed by sequencing of the RT-PCR products. Despite the presences of the P30 transgene constructs and transcripts, no recombinant P30 protein could be detected with SDS-PAGE (Figures 3- 26 to 3- 29) or western blot analysis (Figures 3- 30 to 3- 33) in any P30 transformant. Thus there might be no or very low level of P30 expression (not detectable by the methods employed) in the P30 transformants.

4.2 Further Speculations and Investigations

4.2.1 Other Protein Detection Procedures

Possible degradation of recombinant P30 protein during extraction might be reduced by adding protease inhibitors to the protein extraction buffer and carrying out the extraction procedure on ice.

As the recombinant P30 protein may be very low in amount, isoelectric precipitation, freeze-drying or immunoprecipitation with anti-P30 antiserum could be used to concentrate the crude protein extract before further detection.

4.2.2 *In vitro* Transcription and Translation

Since the problem encountered was at the protein level, *in vitro* transcription and translation was conducted to investigate the translatability of the P30 transcripts. *In vitro* translation products of expected sizes, 25.7 kDa and 24.1 kDa for the P30 proteins with or without a His-tag, respectively, could be detected in the RL system (Figure 3- 35) but not in the WG system (Figure 3- 36). These results indicated that the chimeric P30 genes could be transcribed and translated. Perhaps it is not surprising that the P30 gene can be expressed in the RL system as the *in vitro* reactions were optimized by the manufacturer with the addition of tRNA complement from calf thymus, the use of up to 2 μg of *in vitro* P30 transcript and the absence of protein degradation system. Moreover, the P30 gene had already been

showed to express successfully in animal cells (Kim *et al.*, 1994). However, the failure of the WG system in producing the P30 protein requires further studies.

4.2.3 Gene Silencing at Transcription and/or Post-transcription Levels

For the P30 transformants U1, U2, and U4 harboring the pBI/Ubi-HP, transgene inactivation might have occurred at the transcription and/or post-transcription levels as no P30 transcript could be detected. Since the P30 transgenes were inserted into plant genome in a more or less random fashion, they might integrate into hypermethylated chromosomal regions. This so-called position effect would result in spreading of the methylation pattern to transgenes and inactivating transgene transcription (Prols and Meyer, 1992). Transcriptional gene silencing (TGS), as characterized by reduced transcription of transgene that shares sequence homology in the promoter region in the nucleus and associated with *de novo* methylation of the promoter region of the affected locus (Vaucheret *et al.*, 1998), might be another possibility.

On the other hand, transcription might have occurred but the transcripts were degraded and could not accumulate to a detectable level. In this case, post-transcriptional gene silencing (PTGS), which reduced stable transcript accumulation in the cytoplasm after transcription of the transgene via

sequence-specific RNA degradation and associated with coding sequence methylation (Vaucheret *et al.*, 1998), might occur. PTGS could result from *trans*-interaction between multiple copies of transgenes, between transgenes introduced by successive transformation and between transgenes and homologous host genes (Meyer and Saedler, 1996).

4.2.4 Gene Silencing at Translation and/or Post-translation Levels

Since the P30 gene could be transcribed and the steady-state mRNA level did not seem to be affected, position effect, TGS or PTGS might not be applicable to transformants S, P and PS. Poor P30 expression in these transgenic plants might occur at the translation and/or post-translation levels. Translation of the recombinant P30 transcripts might not be efficient or the resulting proteins were unstable and degraded after translation. Some possible factors contributing to the poor P30 expression in this respect are discussed below.

(A) AUG Context Sequence

The consensus nucleotide sequence surrounding translation initiation codon (AUG) is an important determinant of gene expression level. The effect of the consensus sequence of translation initiation codon in plants had been investigated. Expression cassette containing the GUS coding sequence fused with a plant AUG context sequence (AACAAUGG) under the control of CaMV 35S promoter was

delivered in tobacco mesophyll protoplasts. A three-fold increase in GUS activity was reported as compared to the control (Guerineau *et al.*, 1992). In the present study, this AUG context sequence was not included which might lead to inefficient translation initiation of the P30 sequence.

(B) Codon Usage

In addition to AUG context sequence, the difference in codon usage in tobacco and *T. gondii* might also be a factor contributing to the poor P30 expression. With the exception of Met and Trp, all amino acids are encoded by two to six synonymous codons. Nonrandom usage of synonymous codons is a widespread phenomenon in the majority of species studied to date. This codon bias is suggested to be related to the abundance of corresponding isoaccepting tRNA so as to ensure translation accuracy and efficiency (Ikemura, 1982). The choice of codons has been thought to affect the translation rate. A probable mechanism in explaining the negative influence of the rare codons on translation is that a ribosome may stall when encountering a rare codon as it may take longer time for a rare tRNA to enter the A-site of the ribosome (Yanofsky, 1981).

One example on enhanced gene expression in plants by changing the rare codons to more typical ones is the green fluorescent protein (GFP) from *Aequorea victoria*. By increasing the G+C content of the gene and removing the cryptic

introns and potential polyadenylation sites, one full-size GFP was found in transgenic tobacco while only small and truncated fragments of wild-type GFP were observed in transgenic plants harboring the unmodified gene (Rouwendal *et al.*, 1997).

Based on the assumption that the more frequently used codons have a greater proportion of tRNAs and hence better translation, these frequently used codons are thus more preferred in that organism. As the P30 gene is originated from protozoan, its codon usage is quite different from that of the plants (Table 4- 1). The frequently used codons AUC, GUC, UCG, AGC, CCC, ACA, ACG, GCC, GCG, GAC, UGC (shown in red and shaded in Table 4- 1) in the P30 gene are quite rarely used in tobacco. These differences in codon usage might result in inefficient translation of the P30 sequence in tobacco.

Table 4- 1 Comparison of codon usage frequency in *N. tabacum*, *T. gondii* and the P30 gene

Amino acid	Triplet	Frequency (per 1000) in *N. tabacum*	Frequency (per 1000) in *T. gondii*	Frequency (per 1000) in P30
Phe	UUU	24.1	14.3	12.4
	UUC	17.8	25.4	12.4
Leu	UUA	11.9	4.3	4.1
	UUG	21.9	15.7	16.5
	CUU	24.2	14.7	12.4
	CUC	12.6	23.3	16.5
	CUA	8.9	2.3	4.1
	CUG	10.5	22.2	8.3
Ile	AUU	28.0	17.2	16.5
	AUC	14.0	21.7	20.7
	AUA	12.9	4.1	0
Met	AUG	24.2	21.3	16.5
Val	GUU	27.6	17.4	8.3
	GUC	11.5	27.4	33.1
	GUA	11.1	7.5	8.3
	GUG	16.9	23.7	20.7
Ser	UCU	20.3	13.4	8.3
	UCC	10.5	12.6	12.4
	UCA	17.2	7.8	16.5
	UCG	5.1	14.7	16.5
	AGU	12.7	9.7	16.5
	AGC	10.1	13.7	20.7
Pro	CCU	19.5	13.5	24.8
	CCC	7.0	14.0	16.5
	CCA	20.5	11.4	16.5
	CCG	4.7	15.2	4.1
Thr	ACU	21.5	13.3	28.9
	ACC	10.0	15.7	8.3
	ACA	17.0	14.4	45.5
	ACG	4.4	17.1	33.1
Ala	GCU	32.9	20.3	12.4
	GCC	12.9	20.2	24.8
	GCA	22.9	20.3	24.8
	GCG	5.8	26.2	16.5
Tyr	UAU	17.7	6.7	0
	UAC	13.6	15.6	12.4
***	UAA	1.2	1.1	0
	UAG	0.5	0.5	0
	UGA	1.1	0.8	4.1
His	CAU	13.0	7.1	24.8
	CAC	8.9	12.8	8.3

Amino acid	Triplet	Frequency (per 1000) in *N. tabacum*	Frequency (per 1000) in *T. gondii*	Frequency (per 1000) in P30 gene
Gln	CAA	21.2	12.9	12.4
	CAG	15.4	24.4	16.5
Asn	AAU	27.2	12.2	12.4
	AAC	18.8	23.1	20.7
Lys	AAA	30.6	20.3	37.2
	AAG	33.7	34.2	41.3
Asp	GAU	35.6	18.9	20.7
	GAC	16.9	33.5	37.2
Glu	GAA	34.1	31.4	16.5
	GAG	28.6	36.9	28.9
Cys	UGU	10.2	7.0	16.5
	UGC	8.1	13.4	28.9
Trp	UGG	11.3	10.1	12.4
Arg	CGU	7.7	9.2	0
	CGC	4.0	15.0	0
	CGA	5.2	9.1	0
	CGG	3.7	7.6	0
	AGA	15.1	10.1	4.1
	AGG	12.4	7.5	8.3
Gly	GGU	24.2	20.5	33.1
	GGC	11.9	27.4	4.1
	GGA	24.0	20.3	20.7
	GGG	10.5	12.4	20.7

The frequencies of codon usage are computed from gene sequences available in the GeneBank Release (October 05, 2000). The frequency per 1000 of codons used in each organism or gene was calculated by summing up the numbers of each codon used. Data were based on a total number of 281,365 codons for *N. tabacum*, 60,724 codons for *T. gondii* and 244 codons for the P30 gene with a His-tag. Possible differences of codon usage are shaded and in red. *** represents stop codon.

(C) N-end Rule

In general, the N-terminal sequence of a protein may influence its stability. After the removal of the N-terminal Met by aminopeptidase, the second amino acid becomes the leading one. By the N-end rule, certain amino acids such as Met, Ala, Gly, Ser, Thr, or Val at the N-terminal can confer stabilizing effect to the protein in cytosol. However, when amino acid such as Lys, Arg, His, Phe, Tyr, Trp, Ile, Leu, Asp, Glu, Gln, or Asn is present instead, the proteins will be degraded rapidly by the ubiquitin-dependent proteolytic pathway in the cytoplasm and nucleus (Varshavsky, 1997). In this study, the P30 proteins initiated with Met, and with His (from the His-tag) as the second amino acid, which might render protein instability.

(D) Phaseolin Sorting Signal

Phaseolin is the major storage protein of French bean. According to Frigerio *et al.* (1998), removal of the last four C-terminal amino acids, AFVY (Ala, Phe, Val and Tyr) allows correct trimer formation but causes phaseolin to be secreted into apoplast instead of accumulated in the protein storage vacuoles. The fact that the AFVY tetrapeptide alone is sufficient for sorting protein to the vacuole was further demonstrated by fusing this tetrapeptide to a secreted version of GFP under the control of CaMV 35S promoter. After transfecting this fusion construct into tobacco protoplasts, it was found that the reporter GFP was redirected to and

accumulated in vacuoles (Frigerio *et al.*, 2001).

In this research, the transformants with the pBI/Phas.SP-HP were expected to have their recombinant P30 protein targeted into seed protein bodies since phaseolin SP was present. However, no such AFVY tetrapeptide was included or appeared at the C-terminal of P30. So recombinant P30 protein, at least for the constructs with signal peptide, if produced, would enter ER but not be further sorted to protein bodies. It might be secreted to apoplast and/or being degraded instead.

Chapter 5 Future Perspectives

In this study, the expression of the P30 chimeric genes in tobacco seemed to be blocked at translation and/or post-translation levels. Future studies thus will focus on improving the P30 translation efficiency and protein stability. Two studies have been initiated in this connection: (1) codon modification of the P30 gene to investigate the effect of P30 codon usage on gene expression in plants and at the same time to optimize its expression and (2) the fusion of the P30 gene with a seed protein gene which had shown high and stable expression in transgenic plants, with an aim to stabilize the P30 protein in plants.

5.1 Codon Modification of the P30 Gene

Since codon usage in the P30 gene is quite different from that of plants, it was suspected that this might be a cause of the poor P30 expression. To investigate the effect of P30 codon usage on gene expression in plants, the codons of the P30 gene was modified based on the preferred codons used in two seed storage proteins. Codon usage of lysine-rich protein (LRP) from winged bean (Figure 5- 1; Table 5- 1) and methionine-rich 2S albumin (PN2S) from Paradise nut (Figure 5- 2; Table 5- 2) were chosen as the bases for modification because high expression and stable accumulation (3-10% and 3-15% of total extractable seed proteins for LRP and PN2S, respectively) in transgenic *Arabidopsis* was observed in previous studies (Cheng, 1999; Chen, 2000).

ATGGGTGTTTTCACATATGAGGATGAAACCACTTCACCAGTGGCTCCTGCTATCCTT

TACAAAGCAATAGTTAAAGATGCTGATAACATCTTTCCAAAGGCTGTTGATTCCTTT

AAGAGTGTTGAAATTGTTGAGGGAAATGGTGGTCCTGGAACCATCAAGAAGATCTCT

TTTGTTGAGGATGGGGAAAGCAAGTTTGTGTTGCACAAGATTGAGTCAATTGATGAG

GCTAATTTGGGATACAGCTACAGCATAGTTGGTGGTGCTGCTTTGCCAGACACAGTG

GAGAAGATTACATTTGAGTCCAAATTGAGTGCTGGACCTTCTGGAGGCTCTGTTGGG

AAACTCACTGTGAAATACCAAACCAAAGGAGATGCTGAGCCCAATGAAGAGGAACTC

AAAGTTGGCAAAGCCAAGGGTGATGCTCTCTTCAAGGCTGTTGAGGCTTACCTTTTG

GCCCATCCTGAATACAATTGA

Figure 5- 1 Nucleotide sequence of the LRP cDNA from winged bean
The full length of LRP cDNA is 477 bp. The start (ATG) and stop (TGA) codons are shaded.

ATGGCGAAGATTTCTGTTGTGGCAGGAGCCCTCCTTCTCCTCCTGGTTCTCGGCCAC

GCCACCGCCTTCCGGGCCACCGTCACCACCACAGTGGTGGAGGAGGAGAACCAGGAT

TGCCGCGAGCAGATGCAGAGACAGCAGATGCTCAGCCACTGCCGGAAGTACATGAGA

CAGCAGATGGAGGAGAGCCCGTACCACACCATGCCCAGGCGGGGAATGGAGCCGCAC

ATGAGCGAGTGCTGCGAGCAGCTGGAGGGGATGGACGAGAGCTGCAGATGCGAAGGC

TTAAGGATGATGATAAGGATGATGCAACGGGAGGAGCAACCCCGAGAGGAGCAGAAG

CAAAGGATGATGAGGATGGCCGAGAATATCCCTTCCCGCTGCAACCTCAGTCCCCAG

AGATGCCCCATGGGTAGCTCCATGGCCGGCTTCTGA

Figure 5- 2 Nucleotide sequence of the PN2S cDNA from Paradise nut
The full length of PN2S cDNA is 435 bp. The start (ATG) and stop (TGA) codons are shaded.

Table 5- 1 Summary of codon usage in LRP

Codon	Count	%	Codon	Count	%	Codon	Count	%	Codon	Count	%
TTT-Phe	5	3.14	TCT-Ser	3	1.88	TAT-Tyr	1	0.62	TGT-Cys	0	0.00
TTC-Phe	2	1.25	TCC-Ser	2	1.25	TAC-Tyr	6	3.77	TGC-Cys	0	0.00
TTA-Leu	0	0.00	TCA-Ser	2	1.25	TAA-***	0	0.00	TGA-***	1	0.62
TTG-Leu	5	3.14	TCG-Ser	0	0.00	TAG-***	0	0.00	TGG-Trp	0	0.00
CTT-Leu	2	1.25	CCT-Pro	4	2.51	CAT-His	1	0.62	CGT-Arg	0	0.00
CTC-Leu	3	1.88	CCC-Pro	1	0.62	CAC-His	1	0.62	CGC-Arg	0	0.00
CTA-Leu	0	0.00	CCA-Pro	3	1.88	CAA-Gln	1	0.62	CGA-Arg	0	0.00
CTG-Leu	0	0.00	CCG-Pro	0	0.00	CAG-Gln	0	0.00	CGG-Arg	0	0.00
ATT-Ile	4	2.51	ACT-Thr	2	1.25	AAT-Asn	4	2.51	AGT-Ser	2	1.25
ATC-Ile	4	2.51	ACC-Thr	3	1.88	AAC-Asn	1	0.62	AGC-Ser	3	1.88
ATA-Ile	2	1.25	ACA-Thr	3	1.88	AAA-Lys	8	5.03	AGA-Arg	0	0.00
ATG-Met	1	0.62	ACG-Thr	0	0.00	AAG-Lys	9	5.66	AGG-Arg	0	0.00
GTT-Val	10	6.28	GCT-Ala	12	7.54	GAT-Asp	8	5.03	GGT-Gly	6	3.77
GTC-Val	0	0.00	GCC-Ala	2	1.25	GAC-Asp	1	0.62	GGC-Gly	2	1.25
GTA-Val	0	0.00	GCA-Ala	1	0.62	GAA-Glu	6	3.77	GGA-Gly	6	3.77
GTG-Val	4	2.51	GCG-Ala	0	0.00	GAG-Glu	10	6.28	GGG-Gly	2	1.25

The full length of LRP cDNA is 477 bp with 159 codons and 43.6% G+C content. Codons in preference for each amino acid are boxed.

Table 5- 2 Summary of codon usage in PN2S

Codon	Count	%	Codon	Count	%	Codon	Count	%	Codon	Count	%
TTT-Phe	0	0.00	TCT-Ser	1	0.68	TAT-Tyr	0	0.00	TGT-Cys	0	0.00
TTC-Phe	2	1.37	TCC-Ser	2	1.37	TAC-Tyr	2	1.37	TGC-Cys	8	5.51
TTA-Leu	1	0.68	TCA-Ser	0	0.00	TAA-***	0	0.00	TGA-***	1	0.68
TTG-Leu	0	0.00	TCG-Ser	0	0.00	TAG-***	0	0.00	TGG-Trp	0	0.00
CTT-Leu	1	0.68	CCT-Pro	1	0.68	CAT-His	0	0.00	CGT-Arg	0	0.00
CTC-Leu	6	4.13	CCC-Pro	4	2.75	CAC-His	4	2.75	CGC-Arg	2	1.37
CTA-Leu	0	0.00	CCA-Pro	0	0.00	CAA-Gln	3	2.06	CGA-Arg	1	0.68
CTG-Leu	2	1.37	CCG-Pro	2	1.37	CAG-Gln	10	6.89	CGG-Arg	4	2.75
ATT-Ile	1	0.68	ACT-Thr	0	0.00	AAT-Asn	1	0.68	AGT-Ser	1	0.68
ATC-Ile	1	0.68	ACC-Thr	5	3.44	AAC-Asn	2	1.37	AGC-Ser	5	3.44
ATA-Ile	1	0.68	ACA-Thr	1	0.68	AAA-Lys	0	0.00	AGA-Arg	4	2.75
ATG-Met	18	12.41	ACG-Thr	0	0.00	AAG-Lys	3	2.06	AGG-Arg	5	3.44
GTT-Val	2	1.37	GCT-Ala	0	0.00	GAT-Asp	1	0.68	GGT-Gly	1	0.68
GTC-Val	1	0.68	GCC-Ala	6	4.13	GAC-Asp	1	0.68	GGC-Gly	3	2.06
GTA-Val	0	0.00	GCA-Ala	1	0.68	GAA-Glu	1	0.68	GGA-Gly	2	1.37
GTG-Val	3	2.06	GCG-Ala	1	0.68	GAG-Glu	16	11.03	GGG-Gly	1	0.68

The full length of PN2S cDNA is 435 bp with 145 codons and 60% G+C content. Codons in preference for each amino acid are boxed.

By comparing the frequency of codon usage, priority ranking of synonymous codons for each amino acid was performed (Table 5- 3). It was assumed that the most frequently used codons for each amino acid are the most preferred ones. Codon usage tables for the P30 gene before (OP30) and after (MP30) codon modifications were showed in Tables 5- 4 and 5- 5, respectively. Codons in the P30 gene were optimized to the corresponding preferred ones (summarized in Table 5- 3) with the 1st priority codons as top choices followed by the 2nd priority ones. The modified P30 (MP30) gene was synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA), as illustrated in Figure 5- 3. The length of the MP30 gene (from start to stop codon) is 696 bp. After modification, 96 nucleotides were changed, representing 9.7% modification. A plant AUG context sequence (AACAATGG) was introduced at the 5' end of the gene to enhance translation initiation. According to the N-end rule, Ala was added as the 2nd amino acid to stabilize the protein in cytosol. Two chimeric MP30 genes under the control of the CaMV 35S or phaseolin promoter were constructed and transformed into *Arabidopsis* via *Agrobacterium*-mediated transformation. Expression studies will be carried out in the near future.

Table 5- 3 Summary of codon preference prioritized based on the LRP and PN2S sequences

Amino acid	Triplet		
	1st priority	2nd priority	3rd priority
Phe	TTT / TTC	-	-
Leu	CTC	TTG	CTT /CTG
Ile	ATT / ATC	ATA	-
Met	ATG	-	-
Val	GTT	GTG	-
Ser	AGC	AGT / TCT / TCC	TCA
Pro	CCT / CCC	CCA	CCG
Thr	ACC	ACA	ACT
Ala	GCT	GCC	-
Tyr	TAC	-	-
His	CAC	-	-
Gln	CAA	CAG	-
Asn	AAT / AAC	-	-
Lys	AAA / AAG	-	-
Asp	GAT	GAC	-
Glu	GAG	GAA	-
Cys	TGC	-	-
Trp	TGG	-	-
Arg	CGG / AGA /AGG	CGC	-
Gly	GGC / GGA	GGT	GGG
***	TGA	-	-

Codons of 1st priority are the ones that appear to be most frequently used in LRP and PN2S, and the 2nd as second frequently and so on. *** represents stop codon.

Table 5- 4 Summary of codon usage in the original P30 (OP30)

Codon	Count	%	Codon	Count	%	Codon	Count	%	Codon	Count	%
TTT-Phe	3	1.29	TCT-Ser	2	0.86	TAT-Tyr	0	0.00	TGT-Cys	4	1.72
TTC-Phe	3	1.29	TCC-Ser	3	1.29	TAC-Tyr	3	1.29	TGC-Cys	7	3.01
TTA-Leu	1	0.43	TCA-Ser	4	1.72	TAA-***	0	0.00	TGA-***	1	0.43
TTG-Leu	4	1.72	TCG-Ser	4	1.72	TAG-***	0	0.00	TGG-Trp	3	1.29
CTT-Leu	3	1.29	CCT-Pro	6	2.58	CAT-His	1	0.43	CGT-Arg	0	0.00
CTC-Leu	4	1.72	CCC-Pro	4	1.72	CAC-His	1	0.43	CGC-Arg	0	0.00
CTA-Leu	1	0.43	CCA-Pro	4	1.72	CAA-Gln	3	1.29	CGA-Arg	0	0.00
CTG-Leu	2	0.86	CCG-Pro	1	0.43	CAG-Gln	4	1.72	CGG-Arg	0	0.00
ATT-Ile	4	1.72	ACT-Thr	7	3.01	AAT-Asn	3	1.29	AGT-Ser	4	1.72
ATC-Ile	5	2.15	ACC-Thr	2	0.86	AAC-Asn	5	2.15	AGC-Ser	5	2.15
ATA-Ile	0	0.00	ACA-Thr	11	4.74	AAA-Lys	9	3.87	AGA-Arg	1	0.43
ATG-Met	3	1.29	ACG-Thr	8	3.44	AAG-Lys	9	3.87	AGG-Arg	2	0.86
GTT-Val	2	0.86	GCT-Ala	4	1.72	GAT-Asp	5	2.15	GGT-Gly	8	3.44
GTC-Val	8	3.44	GCC-Ala	5	2.15	GAC-Asp	5	2.15	GGC-Gly	1	0.43
GTA-Val	2	0.86	GCA-Ala	7	3.01	GAA-Glu	4	1.72	GGA-Gly	5	2.15
GTG-Val	5	2.15	GCG-Ala	4	1.72	GAG-Glu	7	3.01	GGG-Gly	6	2.58

The full-length OP30 (without a His-tag and an EK site) gene is 696 bp with 232 codons and 51.5% G+C content.

Table 5- 5 Summary of codon usage in the modified P30 (MP30)

Codon	Count	%	Codon	Count	%	Codon	Count	%	Codon	Count	%
TTT-Phe	3	1.29	TCT-Ser	2	0.86	TAT-Tyr	0	0.00	TGT-Cys	0	0.00
TTC-Phe	3	1.29	TCC-Ser	3	1.29	TAC-Tyr	3	1.29	TGC-Cys	11	4.74
TTA-Leu	0	0.00	TCA-Ser	4	1.72	TAA-***	0	0.00	TGA-***	1	0.43
TTG-Leu	4	1.72	TCG-Ser	0	0.00	TAG-***	0	0.00	TGG-Trp	3	1.29
CTT-Leu	3	1.29	CCT-Pro	6	2.58	CAT-His	0	0.00	CGT-Arg	0	0.00
CTC-Leu	6	2.58	CCC-Pro	4	1.72	CAC-His	2	0.86	CGC-Arg	0	0.00
CTA-Leu	0	0.00	CCA-Pro	4	1.72	CAA-Gln	3	1.29	CGA-Arg	0	0.00
CTG-Leu	2	0.86	CCG-Pro	1	0.43	CAG-Gln	4	1.72	CGG-Arg	0	0.00
ATT-Ile	4	1.72	ACT-Thr	8	3.44	AAT-Asn	3	1.29	AGT-Ser	4	1.72
ATC-Ile	5	2.15	ACC-Thr	13	5.60	AAC-Asn	5	2.15	AGC-Ser	9	3.87
ATA-Ile	0	0.00	ACA-Thr	7	3.01	AAA-Lys	9	3.87	AGA-Arg	1	0.43
ATG-Met	3	1.29	ACG-Thr	0	0.00	AAG-Lys	9	3.87	AGG-Arg	2	0.86
GTT-Val	9	3.87	GCT-Ala	12	5.17	GAT-Asp	8	3.44	GGT-Gly	7	3.01
GTC-Val	0	0.00	GCC-Ala	9	3.87	GAC-Asp	2	0.86	GGC-Gly	4	1.72
GTA-Val	0	0.00	GCA-Ala	0	0.00	GAA-Glu	3	1.29	GGA-Gly	8	3.44
GTG-Val	8	3.44	GCG-Ala	0	0.00	GAG-Glu	8	3.44	GGG-Gly	0	0.00

The full-length modified P30 (MP30) gene is 696 bp with 232 codons and 51.6% G+C content.

KpnI *AccI*

5′ *GGTACC GTCTAC*

AACA ATG **GCT** TTC ACT CTT AAG TGC CCT AAA ACC GCC CTC ACA
GAG CCT CCC ACT CTT GCT TAC TCA CCC AAC AGG CAA ATC TGC
CCA GCC GGT ACT ACC AGT AGC TGC ACA TCA AAG GCT GTG ACC
TTG AGC TCC TTG ATT CCT GAA GCT GAA GAT AGC TGG TGG ACC
GGC GAT TCT GCT AGT CTC GAC ACC GCT GGC ATC AAA CTC ACC
GTT CCA ATC GAG AAG TTC CCC GTG ACA ACC CAG ACT TTT GTG
GTT GGT TGC ATC AAG GGA GAT GAC GCT CAG AGT TGC ATG GTT
ACC GTG ACA GTG CAA GCC AGA GCC TCA AGC GTT GTG AAT AAT
GTT GCT AGG TGC TCC TAC GGT GCC GAT AGC ACT CTT GGT CCT
GTT AAG TTG TCT GCC GAA GGA CCC ACT ACA ATG ACC CTC GTG
TGC GGC AAA GAT GGA GTT AAA GTT CCT CAA GAT AAC AAT CAG
TAC TGC TCC GGA ACC ACA CTG ACT GGT TGC AAC GAG AAG AGC
TTC AAA GAT ATT TTG CCA AAA CTC ACT GAG AAC CCG TGG CAG
GGT AAC GCT AGC AGT GAT AAG GGT GCC ACC CTC ACA ATC AAG
AAA GAG GCT TTT CCA GCC GAG TCA AAA AGC GTT ATT ATT GGA
TGC ACC GGC GGA AGC CCT GAG AAG CAC CAC TGC ACC GTG AAA
CTG GAG TTT GCC GGA GCT GCT GGA TGA

GTATAC GGATCC AAGCTT 3′

AccI *BamHI* *HindIII*

Figure 5- 3 Nucleotide sequence of the modified P30 (MP30) synthetic gene
The length of the MP30 gene (from start to stop codon) is 696 bp. Sixty-seven nucleotides (in red) were changed for codon optimization, representing 9.7% modification. Restriction sites (in italic) are present at the 5' (KpnI and AccI) and 3' (AccI, BamHI and HindIII) ends for subcloning purpose. A plant AUG context sequence (AACAATGG) is introduced (underlined) to enhance translation initiation. Sequence for amino acid Ala (GCT in bold) is added as the 2nd amino acid to stabilize the protein in cytosol.

5.2 Fusion of the P30 Gene with the LRP Gene

Lysine-rich protein (LRP) is a seed protein from winged bean. It contains high lysine content with unknown biological function. According to Cheng (1999), the LRP protein could be expressed and stably accumulated at high level in the seed cytosol of transgenic *Arabidopsis* under the control of phaseolin regulatory sequences. The LRP gene itself may contain some protein-stabilizing signals that would be useful in enhancing the expression of the P30 gene in plants. To test and make use of these possible signals, we plan to fuse the P30 gene with the LRP gene.

Secondary structure of LRP was first predicated from its coding sequence. The mid region of the polypeptide is of loose random coil conformation. The P30 gene, with or without codon modification, will be inserted into this region to minimize its affect on the overall conformation of the fusion protein. Chimeric LRP-P30 genes regulated by the phaseolin promoter and terminator will be transformed into *Arabidopsis* for further expression analysis.

Chapter 6 Conclusion

To test whether plants can be used as bioreactors to produce high quality *T. gondii* surface antigen P30 in bulk amount, the gene encoding this antigen, under the control of different promoters, had been introduced into model plant tobacco. Successful integration of transgenes into tobacco genome was confirmed by genomic PCR and Southern blot analysis. The P30 transcripts were also detected by RT-PCR and northern blot analysis of the P30 transformants. However, no recombinant P30 protein could be detected by SDS-PAGE and western blot analysis. Possible reasons for poor P30 expression in plants may be sited at translation and/ or post-translation levels. Two studies, focusing on improving translation efficiency and protein stability, through codon modification and fusion protein strategy, have been initiated.

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